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A Comparison of Effect of Caronamide and Benzoic Acid on Penicillin Plasma Concentrations.

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The excretion of penicillin by the renal tubules can be inhibited by diodrast,[1,2,3] para-aminohippuric acid,[3,4,5] benzoic acid and sodium benzoate,[6,7,8] and caronamide.[9,10,11] Although both diodrast and para-aminohippuric acid have been used clinically to increase penicillin plasma concentrations, the necessity of administering intravenously large quantities of the drugs has limited their general usefulness. Benzoic acid, sodium benzoate and caronamide can be administered orally and may therefore be more widely used in conjunction with penicillin therapy. Since no comparison between the effects of benzoic acid and the new compound, caronamide, has appeared in the literature, such a comparison is here reported.

Plan of Study. Seven individuals who were afebrile and without evidence of cardiac, renal, or hepatic dysfunction, were selected for this study. Each patient was studied during 3 periods and served as his own control. Hospital routine was not modified and no effort was made to limit the intake of food or fluid.

In order that assayable plasma concentrations of penicillin might be anticipated during the entire 3 hour period over which dose response curves were obtained, intramuscular doses of 200,000 units were employed. This dose was dissolved in 2 cc of sterile saline and the sodium salt of penicillin G‡ was used exclusively. The penicillin dose given immediately before the determination of peni-

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"Staticin" caronamide (4'-carboxyphenylmethanesulfonanilide) used in this investigation was supplied through the courtesy of Sharp and Dohme, Inc.

‡ Commercial Solvents Corporation, Lot No. 46080801, expiration date October, 1949, potency 1572 units/mg.

1 Rammelkamp, C. H., and Bradley, S. W., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 30.

2 Mokotoff, R., Brams, W., Katz, L. N., and Howell, K. M., *Am. J. Med. Sci.*, 1946, **211**, 395.

3 Avery, N. L., Jr., Mayer, O. B., and Nelson, R. C., *Ann. Int. Med.*, 1946, **24**, 900.

4 Beyer, K. H., Flippin, H., Verwey, W. F., and Woodward, R., *J. A. M. A.*, 1944, **126**, 1007.

5 Loewe, L., Rosenblatt, P., and Alture-Werber, E., *Am. Heart J.*, 1946, **32**, 327.

6 Spaulding, E. H., Bondi, A., Jr., and Early, E., *Science*, 1947, **105**, 210.

7 Bronfenbrenner, J., and Favour, C. B., *Science*, 1945, **101**, 673.

8 Bohls, S. W., Cook, E. B. M., and Potter, R. T., *J. Ven. Dis. Inf.*, 1946, **27**, 69.

9 Beyer, K. H., *Science*, 1947, **105**, 94.

10 Crosson, J. W., Boger, W. P., Shaw, C. C., and Miller, A. K., *J. A. M. A.*, 1947, **134**, 1528.

11 Boger, W. P., Kay, C. F., Eisman, S. H., and Yeoman, E. E., *Am. J. Med. Sci.*, in press.

cillin dose response curves was injected into the right deltoid region by one of the authors and massage of the injection site was avoided.

The pattern of patient study was as follows: *Control day.* An intramuscular injection of 200,000 units of penicillin at 6 and 9 A. M. Following the 9 o'clock injection into the right deltoid region, blood specimens for penicillin assay were obtained at intervals of 15 minutes, 30 minutes, 1 hour, 2 hours and 3 hours (this schedule of sampling is referred to as a dose response curve). *Benzoic acid treatment day.* Each patient was given benzoic acid every 3 hours for 24 hours before a dose response curve was determined. At the same time that the 6 and 9 A. M. doses of benzoic acid were given, an intramuscular injection of 200,000 units of penicillin was administered. Following the 9 o'clock penicillin and benzoic acid medication a dose response curve was obtained. Benzoic acid was administered in 0.5 g gelatin capsules; patients JJ, PR, JC and JA received 2 g every three hours. Patients SB, JG and MV received 3 g every 3 hours. *Caronamide treatment day.* This was similar to the benzoic acid treatment day except that caronamide in 0.5 g tablets was administered in place of benzoic acid.

It should be pointed out that, (a) in all instances at least a three day interval intervened between the benzoic acid and caronamide treatment days; (b) in the case of patients JJ, PR, JC and JA the administration of benzoic acid preceded that of caronamide, whereas patients SB, JG and MV received caronamide before benzoic acid; and (c) patients JJ and SB were incompletely studied so that a caronamide treatment day is lacking for JJ and a benzoic acid treatment day is lacking for SB.

Methods. Blood specimens for penicillin assay were drawn into sterile syringes containing 1 cc of 0.4% sodium citrate solution and were promptly transferred to graduated 15 cc centrifuge tubes. The specimens were centrifuged for 15 minutes at 1500 rpm and the total volume as well as the packed cell volume were recorded. The supernatant plasma was harvested and refrigerated in contact with ice and remained so until penicillin assay on the same or subsequent day. Assays were done by the modified [12] serial dilution method of Rammelkamp using a Group A hemolytic streptococcus as the test organism. All penicillin values recorded in Fig. 1 are corrected for the dilution factor introduced by use of the citrate anticoagulant.

Discussion. It is apparent from the data shown in Fig. 1 that caronamide had a striking effect upon penicillin plasma concentrations when administered orally in doses of 2 or 3 g every 3 hours. Equally apparent is the lack of any significant effect of benzoic acid when given in doses of 2 or 3 g every 3 hours. Six patients showed a marked increase of penicillin plasma concentrations in response to caronamide therapy and 6 patients failed to show any favorable response to benzoic acid treatment. The individual responses to caronamide and to benzoic acid were quite uniform but the averaged data are most striking (Fig. 1). Whereas the benzoic acid dose response curve corresponds exactly to that obtained during the control period, the dose response curve modified by caronamide shows fold increases: at 15 minutes—1.67, 30 minutes—3.57, 1 hr.—3.83, 2 hr.—5.35 and 3 hr.—11.68 (average 5.22) times the control values. The enhancement effects noted in this study are in good agreement with those previously noted. [10, 11]

That benzoic acid had little effect on penicillin plasma concentrations if patients were permitted to have an unrestricted diet was noted by Bronfenbrenner and Favour.[7] These authors sharply restricted fluid and salt intake so that "the urine volume commonly fell to 400 to 600 cc in 24 hours," and gave 2.5 g of benzoic acid in capsules every 4 hours. On this regimen, enhancement effects were demonstrated and were ascribed to benzoic acid.

The patients studied in our series were given food and fluid ad libitum and doses of 16 and 24 g of benzoic acid per day. No elevation of penicillin plasma concentrations

[12] Beyer, K. H., Peters, L., Woodward, Roland, and Verwey, W. F., *J. Pharm. and Exp. Therap.*, 1944, **82**, 312.

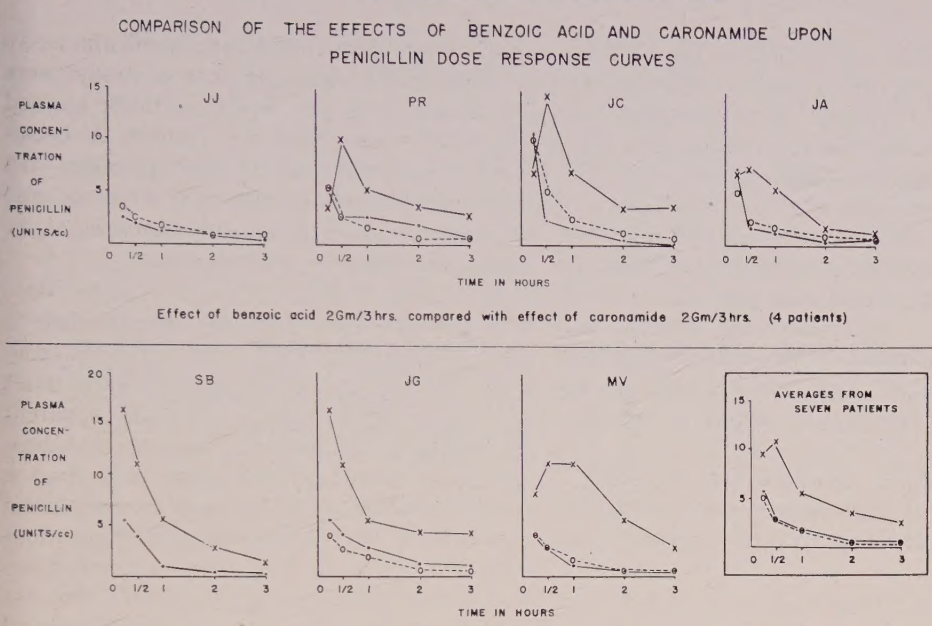

FIG. 1.

was observed. It was suggested that the results reported by Bronfenbrenner and Favour are largely attributable to fluid and salt restriction. The desirability of lowering the daily urinary output to as little as 400 to 600 cc is open to question, especially if patients are suffering from febrile illness. If, however, there is no contraindication to fluid restriction, it can be anticipated that the enhancing effect of dehydration could be superimposed upon the effect of caronamide herein reported.

Spaulding, Bondi and Early[6] reported favorably upon the effect of sodium benzoate in elevating plasma concentrations following the oral administration of penicillin, unrestricted fluid and food intake, and a small dose of sodium benzoate. Bronfenbrenner and Favour indicated that, "on the normal diet, 2.5 g of benzoic acid equalled 6 g of sodium benzoate in raising serum penicillin level." Thus, on an unrestricted diet, Spaulding, Bondi and Early observed a positive effect of a small dose of sodium benzoate, whereas Bronfenbrenner and Favour using a larger dose of benzoic acid, which they regard as a more effective drug than sodium benzoate, observed only a slight effect.

The prolonged enhancing effect of sodium benzoate reported by Bohls, Cook and Potter[8] has been questioned by Spaulding, Bondi and Early on the basis of certain shortcomings of the penicillin assay methods used.

It is quite clear that the few data available on the effects of benzoic acid and sodium benzoate do not permit final evaluation of these two agents as adjuncts to penicillin therapy. If, as indicated by Bronfenbrenner and Favour, restriction of fluid is required to demonstrate an enhancing effect of benzoic acid, use of this agent would have certain disadvantages in the treatment of febrile diseases.

Hippuric acid is formed both in the liver and kidneys by the conjugation of benzoic acid and glycine. Although it is postulated that the hippuric acid thus formed acts in a manner similar to para-aminohippuric acid, it is extremely doubtful whether the amount of circulating hippuric acid following benzoic acid administration is sufficient to inhibit by "mass action" the tubular excretion of penicillin. It is conceivable that benzoic acid may be conjugated in the kidneys and give rise to a "local" high hippuric acid concentration, but this is not subject to proof at present.

In the light of the data here reported, it is our opinion that benzoic acid has no effect upon penicillin plasma concentrations when given in the amounts described.

Conclusions. Benzoic acid given in doses of 16 and 24 g per day to patients on unrestricted diets failed to increase penicillin plasma concentrations. Caronamide given to the same patients in the same doses showed a uniform and marked enhancing effect, averaging five times the control values.

The authors wish to express their indebtedness to Miss Elizabeth Hughes, Department of Bacteriology, Sharp & Dohme, Inc., Glenolden, Pa. who was responsible for all penicillin assays herein reported, and to Doctors Wm. H. Erb, Joseph C. Yaskin, A. Ornsteen, and Sherman F. Gilpin of the staff of the Philadelphia General Hospital, Philadelphia, Pa. for their courtesy in allowing us to use their patients for these studies.

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Further Studies of Effect of Sulphur Compounds on Production of Diabetes with Alloxan.

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In previous publications [1, 2] it was reported that the intravenous injection of large doses of cysteine, glutathione, or thioglycolic acid completely protected rats against a diabetogenic dose of alloxan (40 mg/kg). This protection was observed only when the sulfhydryl compound was administered just prior to the alloxan, or within one minute after the alloxan injection. However if a large dose of cysteine was given 3 or more minutes following the alloxan, no significant protection was observed. It was further suggested that these extraneously administered compounds protected sulfhydryl enzymes from inactivation by alloxan. In this connection it is of interest to note that ascorbic acid, a compound which is reported to decrease the sulfhydryl groups in the body [3] potentiates the effect of alloxan.[4] The more effective production of diabetes in the starved rat [5] may also be related to variation in the glutathione and cysteine content of the body.

Since it was suggested [2] that alloxan may produce its diabetogenic effect by inactivating essential sulfhydryl groups of enzymes, it was desired to test other compounds with known sylfhydryl reactivating effects. The inhibiting effect of arsenic and cadmium on sulfhydryl enzymes has been reported by Peters [6] to be completely reversed by dimercaptopropanol, British Anti-Lewisite, (BAL).

1 Lazarow, A., *Anat. Rec.*, 1945, **91**, 24.

2 Lazarow, A., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 441.

3 Prunty, F. T. G., and Vass, C. C. N., *Biochem. J.*, 1943, **37**, 506.

4 Levey, S., and Suter, B., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 341.

5 Kass, E. H., and Waisbrin, B. A., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 303.

6 Peters, R. A., Stocken, L. A., and Thompson, R. H. S., *Nature, London*, 1945, **156**, 616.

It was desirable to test this compound (BAL) for its antagonistic effect against alloxan diabetes. Also a number of non-sulfhydryl sulphur compounds was tested. Preliminary results have been reported.[7]

Methods. The protection experiments were carried out in the same manner as those previously reported.[2] Alloxan and the protective agent were administered intravenously, using separate tail veins for each injection. To facilitate the intravenous injection, vasodilatation was induced by placing the rats in a 56°C oven for one to several minutes. A 2% solution of alloxan monohydrate was used. The BAL was administered in doses of .16-1.00 mM/kg. The various dilutions of the BAL were prepared in .85% sodium chloride and were injected within 5 minutes of the time of preparation. In other experiments methionine was also used in doses of 2.9 mM/kg. A solution containing 0.25 mM of methionine per cc was injected in doses of 1.15 cc for each 100 g of body weight. This solution is near saturation. Thiourea was also used in doses of 7.5 mM/kg. One cc of a solution containing .75 mM per cc was used for each 100 g of body weight. The solution of thiourea had a pH of about 6.6, and was not neutralized, inasmuch as minute amounts of N/100 sodium hydroxide brings the pH to neutrality. Serial blood sugars were taken at 0, 3, 8, 24, 48, and 72 hours. The blood was obtained by cutting the tip of the tail, the rat being heated to facilitate bleeding; and the sugars were determined by the Folin-Malmros micro method.[8]

Results. The effects of administration of various sulphur compounds are given in Table I. The per cent of animals with diabetes are given for each group, and the average 48-hour blood sugars are listed for the diabetic and non-diabetic animals. An animal was considered diabetic if the 48-hour blood sugar was greater than 200 mg per 100 cc; or if the typical blood sugar response was observed following the injection of alloxan. All of 15 animals, injected with alloxan in doses of 40

TABLE I.

Effect of Various Sulphur Compounds on Production of Diabetes with Alloxan. Both alloxan and the protective agent were given intravenously.

Protective agent	No. of animals	Dose of alloxan mg/kg	Dose of protective agent mM/kg	Substance given first	Time between injections minutes	% diabetic	Avg 48-hr blood sugar	
							Diabetic mg/100 cc	Nondiabetic mg/100 cc
None	15	40	0	0	—	100	444	—
BAL	4	40	.16	BAL	1-2	50	396	154
,,	2	40	.36	,,	1-2	0	—	150
,,	2	40	.72	,,	1-2	0	—	148
,,	2	40	1.00	,,	1-2	Dead	—	—
,,	4	0	.16	—	—	—	—	154
,,	1	0	.36	—	—	—	—	150
,,	2	0	.72	—	—	—	—	140
,,	1	0	1.00	—	—	Dead	—	—
,,	4	40	.16	Alloxan	5	100	404	—
,,	6	40	.75	Alloxan	5	100	403	—
Methionine	4	40	2.9	Methionine	1-2	100	412	—
Thiourea	3	40	7.5	Thiourea	1-2	100	408	—
,,	4	40	7.5	,,	1-2	Death 1-2 hr	—	—
,,	4	0	7.5	—	—	0	—	164

[7] Lazarow, A., *Anat. Rec.*, 1947, **97**, 37.

[8] Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, **83**, 115.

mg/kg, developed diabetes. The average 48-hour blood sugar was 444 mg per 100 cc. When BAL was injected in doses of .16-1.0 mM/kg, immediately preceding a diabetogenic dose of alloxan, the response varied with the amount of BAL administered. Two of 4 rats were protected at a dose of .16 mM/kg of BAL. All animals were protected when the dose was .36-.72; whereas, at a dose of 1.0 mM/kg, the animals died within 2 to 6 hours. BAL alone produced no significant effect in doses of .16-.72 mM/kg, but one animal given a dose of 1.0 mM/kg also died. When the BAL was injected 5 minutes after the alloxan administration, no protection was observed, even with a dose of 0.75 mM/kg which is near the toxic dose for rats.

Methionine, in doses of 2.9 mM/kg (or thiourea in doses of 7.5 mM/kg) did not modify the diabetogenic dose of alloxan. Some animals given thiourea, in doses of 7.5 mM/kg, preceding a diabetogenic dose of alloxan, died within the first 1 to 2 hours, whereas all animals given thiourea without alloxan survived.

Discussion. Mole per mole, BAL, which is a disulfhydryl compound, is at least twice as effective in protecting rats against alloxan diabetes, as is cysteine or glutathione. Doses of .16 mM/kg of BAL protected half of the rats, and doses of .36 mM/kg protected all the rats. With cysteine doses of 1.0 mM/kg, it was previously reported [2] that 12% of the animals became diabetic; whereas, with a dose of .5 mM/kg, 66% of the rats became diabetic. However, allowing for the 2 SH groups in BAL, this compound is at least as effective per mole of SH as is cysteine. The small number of animals used in the BAL experiments do not permit more quantitative comparisons.

It will be noted that the diabetogenic effect of alloxan cannot be modified by BAL when the latter compound is given 5 minutes after the alloxan. This is in marked contrast to the effect of BAL on arsenic and cadmium inactivation of sulfhydryl compounds. Peters [6] has reported that BAL is effective when administered after the heavy metal poisoning has already taken place. Thus if the diabetogenic effect of alloxan is due to inactivation of the sulfhydryl groups of essential enzymes, these sulfhydryl groups cannot be reactivated by BAL. Very little information is available as to the mechanism of inactivation of sulfhydryl groups which takes place with alloxan. If the inactivation consists of merely oxidizing SH groups to S-S groups, one might expect BAL, cysteine, and glutathione to reactivate these groups. However, if the alloxan formed an addition product with the SH groups of enzymes, (as do quinones) this type of inactivation would not be expected to be reversed by glutathione or BAL. Further studies on the combination of alloxan with SH groups are needed.

A complete and irreversible inactivation of the sulfhydryl groups of the beta cells in the pancreas may be requisite to the production of diabetes with alloxan. Reversible sulfhydryl inactivators, such as arsenic, cadmium, etc., might not be expected to produce diabetes because of their reversibility.

The selective inactivation of essential sulfhydryl groups in the beta cells could be a result of a low glutathione content. Since glutathione can protect sulfhydryl enzymes from inactivation, the local concentration of glutathione might determine the sensitivity of cells to sulfhydryl inactivators. Unfortunately, the glutathione content of the beta cells in the islets has not yet been determined.

Inasmuch as alloxan produces its diabetogenic effect within the first 5 minutes of injection, the amount of alloxan reaching the beta cells within this time is a critical factor. Inasmuch as administered glutathione protects against alloxan diabetes, the glutathione, which normally is present in the beta cells, must also act as a protective factor. When this protective amount of glutathione disappears, the alloxan would then be free to act on the cell enzymes. The blood supply of a tissue will also determine the total amount of alloxan reaching the cells during the critical period following its injection, for in a given time, a highly vascular region, such as the islet tissue, may receive a much greater amount of alloxan than other less vascular tissues.

Methionine was also studied because it is important as a methyl donator, and because it contains sulphur. Unfortunately, the solubility of methionine is limited to about .22 mM per cc at 25° C, and in order to inject a dose of 7.5 mM/kg, each rat (average weight of 350 g) would have to have been injected with about 10 cc of fluid intravenously. Consequently, the dose of methionine used in the present experiment was not greater than 2.9 mM/kg. At this dose, no effect was observed; whereas, at corresponding doses of cysteine and glutathione,[2] 100% protection was noted. Thiourea was also studied because of its sulphur content, inasmuch as its sulphur is not in the SH form. Although thiourea did not protect against alloxan damage, some animals given thiourea and alloxan died within the first 1-2 hours. Thiourea alone was not toxic. The mechanism of death in these animals given both thiourea and alloxan has not been determined.

Conclusion. BAL, in doses of .36-.72 mM/kg completely protected rats against a diabetogenic dose of alloxan (40 mg/kg intravenously) when the protective agent was given immediately preceding the alloxan injection. However, no protection was observed when the disulfhydryl compound was given 5 minutes following the alloxan. Methionine, in doses of 2.9 mM/kg, and thiourea, in doses of 7.5 mM/kg, injected immediately preceding a diabetogenic dose of alloxan did not alter the course of alloxan diabetes.

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The Relation of Pteroylglutamic Acid to Metal-Porphyrin Enzymes.*

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Data was reported recently [1, 2] and interpreted as suggesting that pteroylglutamic acid (PGA) and its conjugates are involved in the synthesis of the porphyrin portions of metal-porphyrin enzymes. This communication offers additional data in support of that hypothesis.

For the experiments recorded here the organism *Streptococcus faecalis* (8043) was grown in the usual pteroylglutamic acid assay medium,[3] but with the addition of either potassium cyanide, hydrogen peroxide, or caffeine. The H_2O_2 was diluted, filtered through a Berkefeld filter and added aseptically in the required quantity to sterile culture tubes containing appropriate volumes of medium. KCN was treated similarly, after neutralization with acetic acid. The caffeine was added before autoclaving. The final concentrations of the inhibitors in the culture tubes were as follows: H_2O_2, 0.012%; KCN, 0.05%; caffeine, 0.5% (Table I). After inoculation, turbidity was determined at various intervals with a Coleman spectrophotometer. Responses of the organism to stimulation with varying quantities of PGA as the limiting factor was determined with and without the added inhibitor. As a control procedure, similar experiments were conducted by varying the amount of Ca pantothenate and keep-

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1 Steinkamp, R., Shukers, C. F., Totter, J. R., and Day, P. L., *Fed. Proc.*, 1947, **6**, 295.

2 Totter, J. R., and Sims, E., *Fed. Proc.*, 1947, **6**, 298.

3 Mitchell, H. K., and Snell, E. E., *Univ. of Texas Pub.*, 1941, No. 4137, 36.

TABLE I.
Growth of *Streptococcus faecalis* in Presence of Inhibitors, with Varying Concentrations of Pteroylglutamic Acid (PGA) or Calcium Pantothenate (CaP).

PGA mγ/ml	CaP γ/ml	Incubation time* hr	Caffeine %	KCN %	H_2O_2 %	Optical density
0.2	0.1	24	0.5	0	0	0
0.5	,,	,,	,,	,,	,,	.04
1.0	,,	,,	,,	,,	,,	.11
5.0	,,	,,	,,	,,	,,	.19
10.0	,,	,,	,,	,,	,,	.25
100.0	,,	,,	,,	,,	,,	.55
10	0.01	48	0.5	0	0	.48
10	0.5	,,	,,	,,	,,	.35
10	1.0	,,	,,	,,	,,	.55
10	5.0	,,	,,	,,	,,	.48
10	0.2	24	0	0.05	0	.36
10	0.5	,,	,,	,,	,,	.46
10	1.0	,,	,,	,,	,,	.48
10	5.0	,,	,,	,,	,,	.43
0.2	0.1	32	0	0.05	0	.01
0.5	,,	,,	,,	,,	,,	.07
1.0	,,	,,	,,	,,	,,	.09
5.0	,,	,,	,,	,,	,,	.51
10.0	,,	,,	,,	,,	,,	.55
100.0	,,	,,	,,	,,	,,	.66
0.2	0.1	48	0	0	0.012	.02
0.5	,,	,,	,,	,,	,,	.00
1.0	,,	,,	,,	,,	,,	.06
5.0	,,	,,	,,	,,	,,	.15
10.0	,,	,,	,,	,,	,,	.17
100.0	,,	,,	,,	,,	,,	.67

* The time required for initiation of growth varies with inoculum size, concentration of inhibitor, and concentration of PGA. The incubation times given were chosen so that growth values were intermediate between no growth and complete growth. In the absence of inhibitor, complete growth (optical density = 0.77) was reached at all concentrations of PGA above 1.0 mγ/ml in 18 hours.

ing the PGA concentration constant.

Table I gives representative values for the growth of the organisms (optical density) at appropriate times. It may be seen that the inhibition induced by KCN, H_2O_2, or caffeine was partially reversed by the addition of large quantities of PGA. On the other hand the addition of Ca pantothenate above the normal requirement for this factor was without beneficial effect. It should be pointed out that in the presence of H_2O_2 low concentrations of PGA eventually permitted nearly complete growth, indicating that there was little or no destruction of the vitamin by the peroxide.

Probably the only biochemical property the three inhibitors have in common is their relation to catalase, peroxidase, cytochrome, cytochrome oxidase, and perhaps other metal-porphyrin systems; it therefore seems logical to conclude that PGA acts by stimulating the production of extra quantities of metal-porphyrin enzymes.

The molarity of KCN required to bring about complete inhibition is of the same order of magnitude as that required to reduce the oxidation potential of hemin to a minimum, as shown by Barron.[4]

Keilin has studied the effect of caffeine on hematins and concluded that the purine forms complexes with porphyrin.[5] According to Cheyney [6] the inhibitory action of caffeine

[4] Barron, E. S. G., *J. Biol. Chem.*, 1937, **121**, 285.

[5] Keilin, J., *Biochem. J.*, 1943, **37**, 281.

[6] Cheyney, R. H., *J. Gen. Physiol.*, 1946, **29**, 63.

on fertilized *Arbacia* egg respiration is mediated through the cytochrome-cytochrome oxidase system. The percentage of caffeine herein found to inhibit *S. faecalis* is very similar to that found by Cheyney to inhibit cell division in *Arbacia*. These considerations would appear to offer strong support to the hypothesis suggested in this and earlier communications.

Summary. *Streptococcus faecalis* was grown in the usual PGA-deficient medium containing graded amounts of PGA, to which either 0.05% potassium cyanide, 0.5% caffeine, or 0.012% hydrogen peroxide was added as an inhibitor. The inhibition induced by any of these three substances was partially reversed by large amounts of PGA. In control experiments where the Ca pantothenate content of the medium was varied but the PGA content kept constant, no such reversal of inhibition was found even with high levels of Ca pantothenate. These data lend additional support to the suggestion that PGA is involved in the synthesis of the porphyrin portions of metal-porphyrin enzymes.

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Habitat of *Endameba buccalis* in Lesions of Periodontoclasia.*

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Many years ago Johns and I[1] showed that *Endameba buccalis* are most numerous at the very bottom of the pyorrhoea pocket. Later Kofoid[2] substantiated, and extended the application of this observation.

Studies of amebae in periodontoclasia have been based largely upon material taken from the lesions around and between teeth *in situ*. Although dental literature contains large numbers of illustrations of sections of teeth, including the periodontal tissues in all stages of periodontoclasia, the amebae present usually have been overlooked. Probably this has resulted from the fact that they are located within the bacterial film on the tooth and are not easily recognized in sections prepared in the usual way. Kofoid and Hinshaw[3] reported the distribution of the amebae found at different levels in relation to the calculus in sections of two incisors removed at biopsy.

More recently, employing an entirely different method, I have been able to ascertain, more accurately, the location and habitat of the parasite in these lesions. The method consists essentially of microscopic study of material removed, under the dissecting microscope, with delicate micro-instruments, from different locations on extracted teeth which have been stained to facilitate indentification of the structures and material present.

Elsewhere I[4] have described a previously unrecognized demonstrable line on extracted teeth which indicates the location of the outer border of the epithelial attachment. It is called the "zone of disintegrating epithelial-attachment cuticle" or zdeac. This line not only indicates the location of the outer border of the epithelial attachment, but it also accurately indicates the location, on the tooth, of the very bottom of the periodontoclasia lesion. With the zdeac as a guide, small particles of the soft bacterial film material can be picked from any selected areas and

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1 Bass, C. C., and Johns, F. M., *J. A. M. A.*, 1915, **64**, 553.

2 Kofoid, C. A., *J. Paras.*, 1929, **15**, 151.

3 Kofoid, C. A., and Hinshaw, H. C., *J. D. Res.*, 1929, **8**, 446.

4 Bass, C. C., *J. D. Res.*, 1946, **25**, 401.

locations at and near the bottom of the lesion.

Technic. The equipment and technical procedures previously described [4] are required. Safranin (0.5% in water) is satisfactory for staining tooth specimens for the present purpose. Immerse the formalin preserved specimen in the stain 3 to 5 minutes or longer. Wash well in water and examine under the dissecting microscope. The zdeac is not sharply shown on specimens stained with safranin but it can be recognized satisfactorily by one who has previously familiarized himself with this important landmark.

For removal of particles of material for examination under higher magnifications, a suitable micrurgic blade is required. This is made [4] from a very fine needle, the point of which is ground to a thin blade or chisel shape, not more than 0.25 mm wide.

With the specimen in the field of the dissecting microscope and a good light focussed upon it from above, small particles are picked from the thin edge of the soft stained material extending down to, and sometimes overlapping, the outer border of the zdeac. One or more such selected particles are transferred to a droplet of 50% glycerine on a slide and there teased apart, if desired, with the aid of 2 very fine pointed needles. A ¼ size coverglass is put on but not pressed down too hard. The mounted specimen is now ready for examination.

By proper adjustment of the light, different levels around the thin edge of particles and for some distance inward, can be focussed well enough to recognize the amaebae and the filaments and branches of the leptotrichia among which they are imbedded.†

The Periodontoclasia Lesion. The lesion of periodontoclasia consists of a pocket or space ("pyorrhoea pocket") at various locations about the tooth, and may extend all way or only part way around it. There is much variation in the depth of lesions around different teeth, and that of the lesions at different locations around the same tooth.

On one side of the lesion there is an inflamed suppurating surface of epithelial tissue extending from the gingival margin to the bottom of the lesion. On the opposite side is the tooth which is covered with more or less hard calculus. Attached to the calculus and to the tooth near it, not yet covered with calculus, there is a pad of soft bacterial material consisting of a compact mass, of variable thickness, of stems and filaments extending outward towards the space and downward towards the very bottom of the lesion. This latter has been noted recently by Box,[5] not, however, with any reference to amebae present.

The outer part of the pad attached to the tooth consists largely of radiating filaments which protrude at the surface as a thick-set carpet-like pile of growing, branching and fruiting stems. It is possible that the compact portion of the baterial pad attached to the tooth may consist of several different kinds of organisms of this type. However, the fruiting heads on the surface and the stems that can be focussed deeper in conform, in most instances, to *Leptothrix falciformis*. This organism was first described in material from around teeth by Buest [6] and given the name *L. falciformis,* because of the scythe- or blade-shaped conidia produced on the fruiting branches. These conidia or spore bearing curved to straight rods of varying size radiate, at an angle, from the central stalk, which is also surrounded by a large amount of jelly-like material in which the falciforms are imbedded.

Association of the Amebae with Leptotrichia and their Distribution. Intimate association of *E. buccalis* with filamentous bacterial material has been observed.[3,7,8] Good-

† The amebae are imbedded in a mass of bacterial material. This must be stained lightly with a weak stain to permit focussing upon them through the mass.

These amebae have not been generally observed in paraffin or celloidin sections of teeth and parodontal tissues. Perhaps this explains, to some extent, why the habitat of this widely distributed parasite has not been generally recognized in such material heretofore.

5 Box, H. K., *J. Canadian D. A.*, 1947, **13**, 3.

6 Buest, Theo. Von, *Dent. Cos.*, 1908, **50**, 594.

7 Barrett, M. T., *Dent. Cos.*, 1914, **56**, 948.

8 Goodrich, H. P., and Mosely, M., *J. Roy. Mic. Soc.*, 1916, Dec., 513.

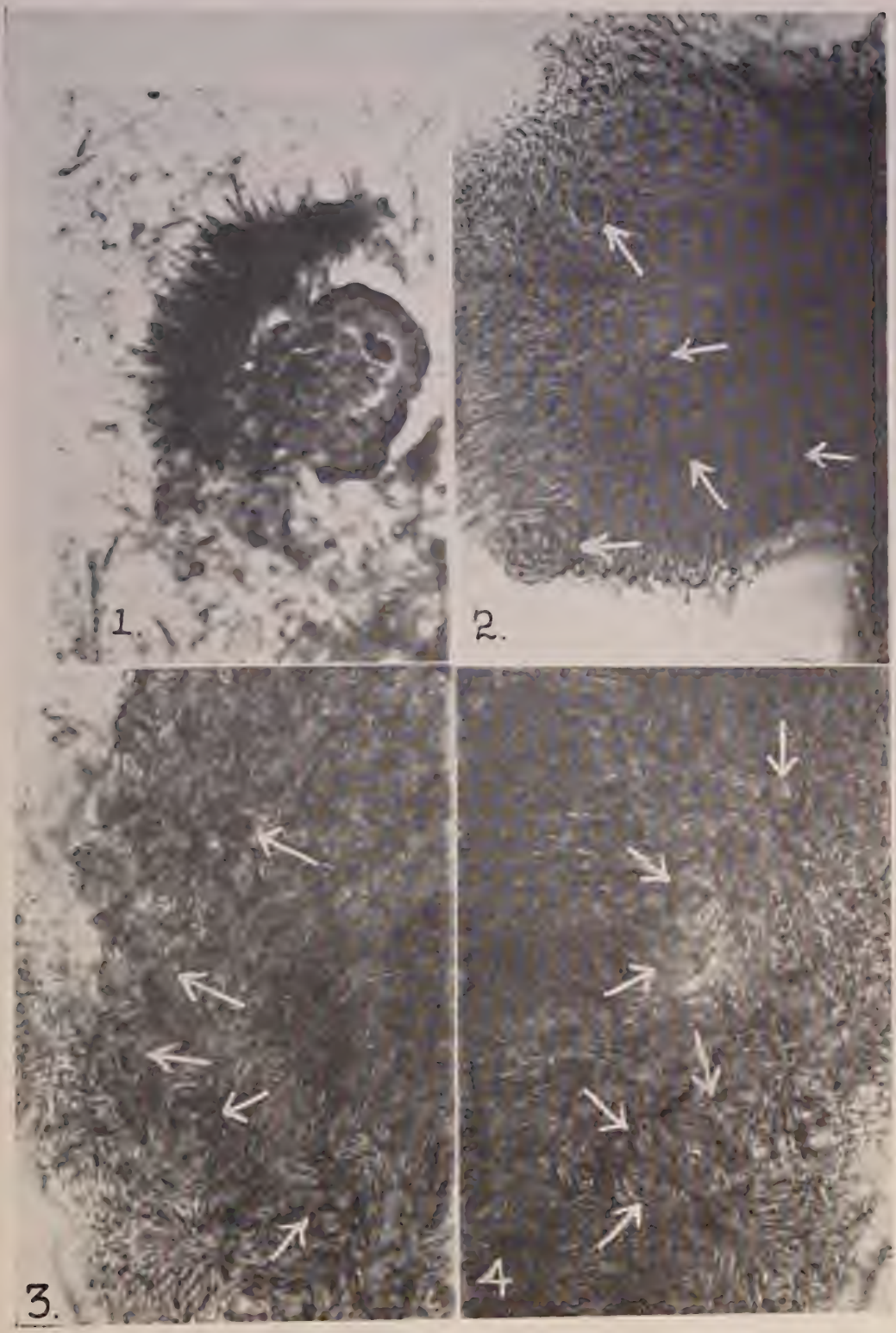

Fig. 1. *E. buccalis* mounted, for comparison, by the side of an isolated fruiting stem of *L. falciformis*. × 1200.

Fig. 2. Outer edge of leptothrix bed; fruiting head covered with spirochetes; arrows point to ameba at edge and others, not sharply focussed, deeper in. × 810.

Fig. 3. Leptothrix bed; arrows point to amebae, partially focussed. × 810.

Fig. 4. Deep leptothrix bed; arrows point to imbedded amebae. × 810.

rich and Mosely[8] found that these amebae *(Entameba gingivalis)* are in greatest numbers on the under side of the tartar ridge. They claim that the parasites do not burrow into the tissues of the gum but often between the terminal branches of the leptotrichia which are found in abundance in the pyorrhoea lesion.

The space between the leptotrichial bed on the one side and the inflamed epithelial wall on the other, contains inflammatory tissue exudate, large numbers of bacteria of many varieties, spirochetes and usually some amebae which have come out from their bed to their feeding ground where there are an abundance of pus cells upon which they feed. After feeding the parasite usually withdraws into the leptothrix bed for safety and protection. Individuals that venture too far away from the bed into the open space are unable to return and are swept out with the pus, especially when it is squeezed out by pressure upon the tooth in chewing, biting, etc.

The parasites are found scattered among the branches and fruiting heads (Fig. 1) of the leptothrix bed. The individual not only burrows between the different elements—stems, filaments, falciforms—making up the outer surface, but apparently they also burrow about in the abundant jelly-like material imbedding these elements (Fig. 2, 3, 4). In studying several hundred such specimens as suggested here, I have often observed several amebae clustered about a leptothrix stalk and especially in the fork where a large stalk apparently divides into 2 smaller ones. For the most part, however, they are found separate and not in direct contact with each other.

Summary. A simple method is given, of collecting material from the area on extracted teeth, which is inhabited by *E. buccalis*.

The habitat of *E. buccalis* is the outer part of the filamentous bacterial film on the tooth, within the periodontoclasia lesion. There they are protected and live, grow and multiply among the strands and fruiting heads of leptotrichia, principally *L. falciformis*.

15966

Creeping Eruption Caused by the Larvae of the Cattle Hookworm *Bunostomum phlebotomum.*

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Several years ago while making a series of inoculations with third stage larvae of the hookworm, *Bunostomum phlebotomum,* and the nodular worm, *Oesophagostomum radiatum,* by placing the larvae on the skin of the calves we frequently noted the appearance of small inflamed spots between our fingers.[1] Some of these spots increased after a few hours to about ¼ inch in diameter, followed after 2 or 3 days by the appearance of a narrow, linear, tortuous eruption which became extended at intervals during the next few days. Following the course of these eruption areas a slightly raised, vesicular line generally developed within a few hours which might be interrupted at points. The entire area became more or less swollen and intensely itchy particularly in the mornings. Within about 2 weeks the surface of the skin was dry, scaly, and the irritation gone. Recently another series of inoculations were undertaken using pure cultures of hookworm larvae[2] and we again noted the appearance of these spots always coincidently with the skin inoculations on the calves.

The following may be taken as a typical

1 Mayhew, R. L., *Cornell Vet.*, 1939, **29**, 367.

2 Mayhew, R. L., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 360.

description of one of these lesions. On October 3, 1946 several lesions developed following the application of larvae to the skin of a calf, one of these being on the interdigital surface of the ring finger on the side adjacent to the middle finger and at the distal joint. This lesion developed to the diameter of ½ inch in 2 or 3 hours, and remained in this condition until the night of October 7, when a narrow, elevated, linear extension about ¼ inch long appeared directed toward the palmar side of the finger. On the night of October 8 a right angle extension about ¼ inch long developed toward the tip of the finger. Between 6 and 9 P. M. on October 9 the eruption area was extended toward the palmar side of the finger about ¾ inch and back almost to the starting point in the form of an irregular oval. On the night of October 10 the eruption area was again extended about ¼ inch toward the tip of the finger. The entire area became swollen and was very itchy especially in the mornings. A slightly raised line, interrupted in places, and becoming vesicular in places after a few hours, developed along the eruption area. This vesicular line possibly represents the path of migration of the larvae. No additional migration was observed and by October 12 the swelling and irritation was beginning to disappear. In about another week the area no longer showed irritation, the swelling was gone, and the outer layer of skin was scaling off along the course of migration.

In our experience there was no evidence that the larvae penetrated by way of the hair follicles since no eruption areas appeared on the back of the fingers or hand where hair follicles are present but always on the relatively thin skin areas between the fingers. Two instances were noted in which larvae penetrated on the palm side of the fingers. One of these was on the palm side of the thumb near the tip and the other between the first and second joints of the left index finger. In each instance the skin had been punctured and slightly torn a day or two before and there was migration over ½ to ¾ inch area.

In a few instances a tingling or prickling sensation was noted. One of these we were able to observe in particular. A drop of the

Fig. 1.
Photograph showing eruption area in which 4 and possibly 5 larvae had penetrated. The linear eruptions of 2 of the larvae extended around on to the palmar side of the finger.

larval suspension was observed to run along between the index and middle finger of the left hand as they were held together, and within a few seconds a distinct prickling sensation was noted. That the calf has similar sensations is indicated by the fact that they have been observed many times to twitch the skin of the area to which the larvae are being applied during the next few minutes after contact with the suspension, and also to switch the tail toward the side on which the larvae had been applied.

The lesions and symptoms are in the main identical with those reported by Kirby-Smith, Dove, and White,[3] and White and Dove,[4] as developing from larvae of the dog hookworm, *Ancylostoma brazilense,* in man, and known as creeping eruption. The principal difference seems to be that the duration of the eruptions and irritation is not so prolonged in the case of the cattle hookworm. In our experience with the cattle hookworm the dura-

[3] Kirby-Smith, J. L., Dove, W. E., and White, G. F., *Arch. Derm. Siph.*, 1926, **13**, 137.

[4] White, G. F., and Dove, W. E., *J. Am. Med. Assn.*, 1928, **90**, 1701.

tion is one to three weeks while that of the dog hookworm is from several weeks to months. We also have the impression that the larvae of the cattle hookworm do not penetrate is as large numbers as those of the dog hookworm. In the instances coming under our observation there was no secondary infection while in the case of the dog hookworm larvae this may develop as a result of the prolonged scratching. There is much similarity to the schistosome dermatitis which has been so extensively studied in recent years by Cort,[5] and his associates. There is, however, no migration in schistosome dermatitis.

[5] Cort, W. W., *Am. J. Hyg.*, 1936, **23**, 349.

15967

Histochemistry XIX. Localization of Alkaline Phosphatase in Normal and Pathological Human Skin.*

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The present study was undertaken in the hope of establishing the histo- and cytologic distribution of alkaline and acid phosphatase and lipase in normal and pathologic human skin. Our results were essentially negative for the acid phosphatase and lipase, so that this report deals with alkaline phosphatase alone. The methods of Gomori[1] for the demonstration of the enzymes were employed throughout. All tissue sections were placed in 0.1 M citrate buffer, pH 4.5-5.0, for 30 minutes before the enzyme staining reaction was applied in order to remove preformed phosphates and other substances which might give a positive reaction. Inasmuch as control experiments on these sections demonstrated no staining whatsoever in the absence of substrate, any staining obtained could be ascribed to enzyme action alone. An incubation period of 12-24 hours in the substrate medium was used. In this study 32 specimens of normal skin, collected from 27 individuals, were selected from the scalp, face, breast, axilla, chest, palm, back, abdomen, penis, scrotum and hip. This selection was made because of the differences in histologic structure of skin from those sources. The 33 pathological specimens were biopsied from 8 cases of eczema, 2 cases of lupus erythematosus, 2 cases of psoriasis, 1 case of papular urticaria, 2 cases of pyogenic granuloma, 15 cases of healing wounds and scars, and 3 cases of acne vulgaris.

In normal skin, alkaline phosphatase activity was demonstrated in all specimens. There was slight staining in the stratum granulosum in agreement with the findings of Bourne and MacKinnon[2] who investigated guinea pig skin. The endothelial walls of the capillaries showed the most uniform and clear cut staining effects as previously observed by Gomori[3] and others. The reaction was most pronounced in the nuclei of the endothelial cells and the nucleoli showed a stronger reaction than the other parts of the nuclei. The hairs and hair follicles, especially the papillae, gave an intense reaction in much the same manner as found for guinea pig skin by Johnson *et al.*,[4] and Bourne and MacKinnon.[2] However, in the present investigation, a

* Aided by a grant from the Medical Research Fund of the Graduate School, University of Minnesota.

[1] Gomori, G., *Am. J. Clin. Path.*, 1946, **16**, 347.

[2] Bourne, G., and MacKinnon, M., *Quart. J. Exp. Physiol.*, 1943, **32**, 1.

[3] Gomori, G., *J. Cell. and Comp. Physiol.*, 1941, **17**, 71.

[4] Johnson, P. L., and Bevelander, G., *Anat. Rec.*, 1946, **95**, 193.

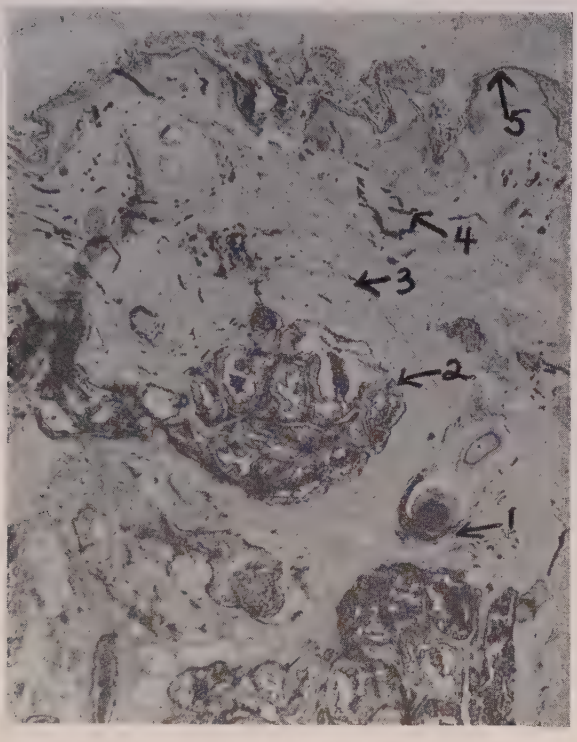

Fig. 1.
Section of axillary skin × 75. Showing slight enzyme activity in stratum granulosum and intense reaction in follicular papilla, capillaries and apocrine glands. (1) Follicular papilla, (2) apocrine glands, (3) sebaceous glands and surrounding vascular bed, (4) capillaries, (5) stratum granulosum.

greater intensity of staining was observed at the base of the follicular papillae which is the site of the nutrient capillary (Fig. 1). This was not previously described in the work on guinea pig skin. The only evidence of alkaline phosphatase activity we were able to demonstrate associated with the sebaceous glands was in the rich capillary bed surrounding them (Fig. 1). This is in contrast to the reports dealing with the work on guinea pigs[2] in which definite staining of sebaceous glands was reported.

Although previous reports have not mentioned alkaline phosphatase in the sweat glands, these glands showed marked activity with some variation in intensity in the loops. The activity in the apocrine glands was more clearly and uniformly demonstrable than in the eccrine glands. In both, the stain was fairly diffuse but it was most prominent at the periphery of the cell and in the nucleus (Fig. 2). The heavier staining of the nucleolus as compared to the rest of the nucleus was most clearly apparent in the apocrine cell (Fig. 3).

Among the dermatologic states found to show no abnormal alkaline phosphatase activity are lupus erythematosus, psoriasis, and papular urticaria. Three specimens from different patients with acne vulgaris showed some diffuse deeper staining involving the lymphocytic infiltrate. The greatest change was seen in scar tissue or tissue showing evidence of new fibroblastic proliferation. In two biopsies obtained a few days after closure of a burn wound the nuclei of the fibroblasts gave a stronger reaction than the cellular cytoplasm. Three older scar tissues (2-8

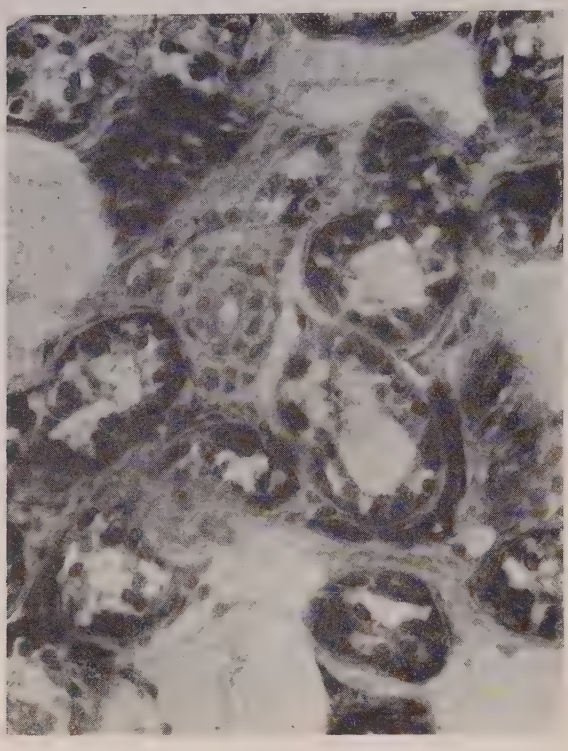

Fig. 2.
Section of eccrine glands × 300. Nuclei, nucleoli and cell walls show positive reactions. The lighter staining sweat duct with its multiple layered cell wall can be seen.

months after healing of an operative wound) showed very definite, though slightly diminished, alkaline phosphatase activity in the fibroblasts (Fig. 4). Still older scars of the same type contained a few scattered groups of fibroblasts giving the positive reaction. These results are in accord with the observations of Fell and Danielli[5] who described the distribution of alkaline phosphatase in the fibroblasts and new collagen fibers of healing experimental wounds in rats. In the same year, Gomori reported phosphatase positive fibroblasts in connective tissue subjacent to newly formed epithelium in fascial transplants of bladder epithelium.[6] In a pyogenic granuloma the new fibroblasts reacted in the same fashion as those in scar tissue. The infiltrate, consisting of polymorphonuclear leucocytes and lymphocytes also gave a positive phosphatase reaction.

A study of the skin in 3 cases of eczema and in 3 cases showing positive allergic skin tests was made. Sections from the sites of positive allergic reactions showed no abnormal change nor did those from relatively acute eczemas. In 3 cases of long standing eczema with infiltration and thickening of the skin the picture was quite different (Fig. 5). Here the phosphatase reaction of the perivascular infiltrate, made up of lymphocytes, monocytes and some plasma cells, was distinctly positive. There was also some diffuse staining of the connective tissue including some of the fibroblasts in the deeper cutis.

Summary. In normal skin, alkaline phosphatase activity was observed in the stratum granulosum, endothelial lining of the capillaries, in hairs, hair follicles, and sweat glands. In the hair follicles, the greatest activity was

[5] Fell, H. B., and Danielli, J. F., *Brit. J. Exp. Path.*, 1943, **24**, 196.

[6] Gomori, G., *Am. J. Path.*, 1943, **2**, 197.

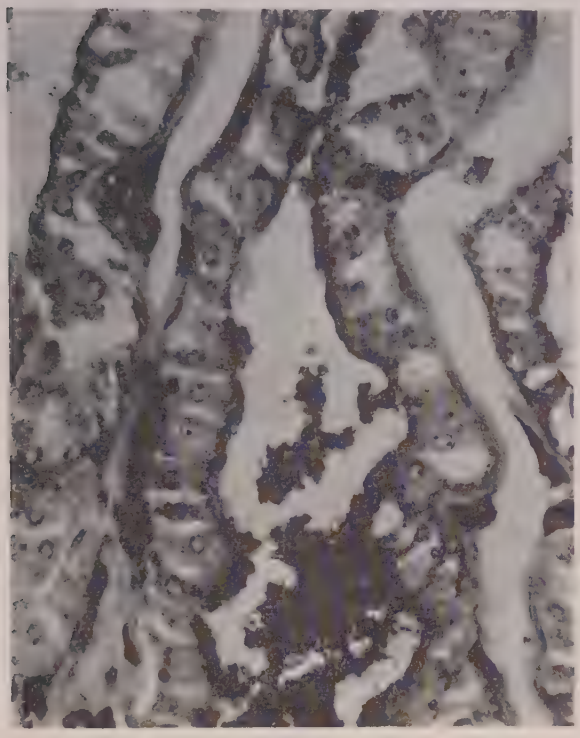

Fig. 3.
Section of apocrine glands × 300. The deeper staining of the nucleoli is apparent.

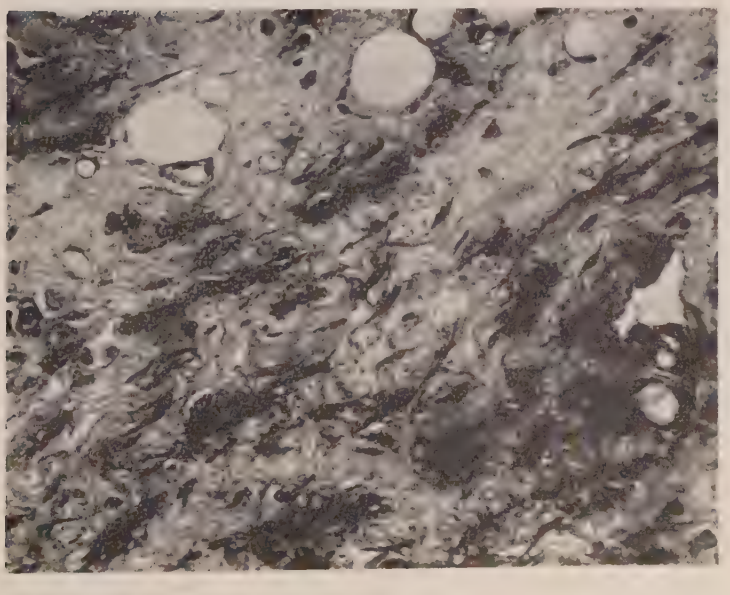

Fig. 4.
Section through a scar (8 mos.) × 150. Enzyme activity pronounced in fibroblasts and connective tissue fibers.

Fig. 5.
Section of chronic eczema. In the deeper cutis, proliferative activity is manifested by the presence of alkaline phosphatase in the new fibroblasts and in the connective tissue fibers. Staining of some of the infiltrate is also shown.

in the papillae. In the endothelial cells and cells of the sweat glands, the enzyme appears to be concentrated at the cell walls and in the nuclei, the nucleoli showing even greater activity than other parts of the nuclei.

Pathologic tissue from cases of lupus erythematosus, psoriasis and papular urticaria showed no significant changes in the enzyme activity.

Alkaline phosphatase activity was found in proliferating fibroblasts in scar tissue, especially in the newly formed scar, and also in the fibroblasts and infiltrate of pyogenic granuloma. The nuclei gave a more intense reaction than the cytoplasm of the cells.

In chronic eczema of long standing, the cellular perivascular infiltrate of lymphocytes, monocytes and plasma cells, as well as the fibroblasts, showed significant alkaline phosphatase activity.

In acne vulgaris, the infiltrate exhibited the enzyme activity.

The authors are indebted to Misses Verna Lorenzoni, Elsa Janda and Mrs. Irene Daniels for technical assistance.

15968

Cobalt Polycythemia. II. Relative Effects of Oral and Subcutaneous Administration of Cobaltous Chloride.

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(Introduced by Arthur A. Hellbaum.)

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In spite of the fact that the efficacy of cobalt salts in stimulating erythropoiesis is well established, cobalt therapy is little used in the treatment of anemia.[1] This is probably due to the fact that many experimenters have given cobalt salts to animals by subcutaneous injection, whereas oral administration should be preferred for human use. Also, there are few data concerning relative toxicity. No data have been recorded concerning the relative effects of cobalt salts on erythrocyte and hemoglobin production when administered orally, in contrast to subcutaneous injection. It has therefore been impossible to evaluate the effects of subcutaneous injection in terms of effective oral dosage. Approximately 4 times as much cobalt is excreted in the urine within the first 72 hours following subcutaneous administration as when the same amount is given orally.[2,3] This difference is apparently related to the difference in rates of absorption.

In order to determine the effects of cobalt on erythrocyte and hemoglobin production under conditions of oral versus parenteral administration, and to establish further the optimal and minimal toxic dosage of this substance, the following experiment was devised. Of 30 albino rats, Sprague-Dawley strain, weighing approximately 250 g each, 24 received cobaltous chloride;* 6, untreated, served as controls. The group of animals to be treated was subdivided into 6 groups of 4 each. Group I received 2.5 mg/kg $CoCl_2 \cdot 6H_2O$ daily by mouth. Group 2 received 2.5 mg/kg daily by subcutaneous injection. Groups 3 and 4 received 10 mg/kg daily, orally and by subcutaneous injection, respectively. Groups 5 and 6 received 40 mg/kg daily, orally and by subcutaneous injection, respectively.

Each dose of cobalt salt was injected subcutaneously in a solution of 0.2ml of distilled water. For oral administration, a solution of the cobalt salt was mixed with equal parts of wheat flour and powdered sugar to form a stiff paste and packed in No. 5 gelatin capsules. Just before administration the capsules were moistened and then inserted far back in the animal's pharynx. This method allowed a careful control of the oral dosage both as to amount and time of administration.

The experiment was continued for 8 weeks. Blood counts and hemoglobin determinations were made at the beginning of the experiment and at 2-week intervals. These data are summarized in Table I. It is apparent that 2.5 mg $CoCl_2 \cdot 6H_2O$/kg/day, given subcutaneously, effected an increase of 30% in the number of erythrocytes per cu mm of blood in 6 weeks. It required approximately 40 mg/kg/day of cobaltous chloride by mouth, or 16 times as much to produce a comparable increase in erythrocytes. Animals which received 10 mg/kg of cobaltous chloride daily by the subcutaneous route presented a more prompt erythrocyte and hemoglobin response. A daily dose of 2.5 mg/kg produced an essentially

1 Stanley, A. J., Hopps, H. C., and Hellbaum, A. A., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 130.

2 Greenberg, D. M., Copp, D. H., and Cuthbertson, E. M., *J. Biol. Chem.*, 1943, **147**, 749.

3 Sheline, G. E., Chaikoff, I. L., and Montgomery, M. L., *Am. J. Physiol.*, 1946, **145**, 285.

* Preparations of $CoCl_2 \cdot 6H_2O$ labeled "Reagent" vary considerably in their content of impurities. At least one such preparation contained sufficient lead to produce lead intoxication if given in amounts described here. We have restricted our use to the preparation marketed by Eimer and Amend and labeled "Cobalt Chloride C. P. $CoCl_2 \cdot 6H_2O$ Mol. wt. 237.95 Cat. no. C-371."

TABLE I.
Relative Effects of Cobaltous Chloride ($CoCl_2 \cdot 6H_2O$) on Production of Erythrocytes and Hemoglobin in Rats when Administered Orally and by Subcutaneous Injection.

No. animals	Daily dose mg/kg Oral	Daily dose mg/kg Subcut.	Erythrocytes in millions/cu mm. Time in weeks 0	2	4	6	8	Hemoglobin in g per 100 ml Time in weeks 0	2	4	6	8
4	2.5		8.84	9.07	8.90	9.56	8.64	16.2	16.2	17.0	17.0	17.2
4		2.5	8.65	9.44	10.33	11.38	11.65	16.1	16.4	19.4	21.8	22.5
4	10.0		9.03	9.76	10.28	10.58	10.40	16.2	16.6	18.2	20.0	20.0
4		10.0	9.36	10.07	12.59	11.98	11.73	16.4	18.2	22.2	22.9	22.4
4	40.0		8.83	9.25	10.05	11.17	11.42	15.9	17.0	18.6	20.8	21.7
4*		40.0	8.84	10.01	—	—	—	15.8	17.8	—	—	—
6	Controls		8.72	9.01	9.51	8.82	9.06	16.0	16.5	16.7	16.8	16.2

* None of these 4 animals survived beyond 8 days due to the toxicity of cobalt in such massive doses. Erythrocyte and hemoglobin determinations were made at the end of 6 days rather than at 2 weeks. The animals receiving the same dose orally survived.

similar effect, but required an additional 2 weeks.

Rats which received 2.5 mg/kg of cobaltous chloride subcutaneously or 40 mg/kg by mouth did not manifest significant toxic effects. Their body weight remained essentially constant, as did that of the controls. Rats which received 10 mg/kg of cobaltous chloride subcutaneously lost an average of 24% of their body weight by the end of 6 weeks. Those animals which received 40 mg/kg daily, subcutaneously, survived 5, 7, 8, and 8 days, respectively.

Summary. The relative effect of parenteral versus oral administration of cobaltous chloride on erythrocyte and hemoglobin formation in rats has been determined. Daily subcutaneous injection of 2.5 mg/kg of body weight, over a period of 6 weeks, resulted in an increase of more than 30% in the number of erythrocytes per cu mm and in grams of hemoglobin per 100 ml of blood. To produce a similar effect from oral administration of cobaltous chloride, within the same period of time, 40 mg/kg of body weight was required. These dosages are without significant toxic effects. Rats which received 10 mg cobaltous chloride/kg daily, subcutaneously, averaged 24% weight loss by the end of 6 weeks. Rats which received 40 mg/kg body weight, subcutaneously, did not survive beyond 8 days.

15969

Inhibition of the Hirst Haemagglutination Reaction by Pneumococcal Extract, Normal Serums, and Blood Cell Esterases.*

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During the course of other studies it was observed that culture filtrates of Type I and Type V pneumococci would inhibit the agglutination of chicken red cells by influenza virus. Stone[1,2] found that lecithinase would

* While the final draft of this paper was being prepared several studies[3-5] were reported before the Society of American Bacteriologists, in Philadelphia, dealing with the nature of agglutinin-inhibiting substances.

[1] Stone, J. D., *Australian J. Exp. Biol. and Med. Sci.*, 1946, **24**, 191.

[2] Stone, J. D., *Australian J. Exp. Biol. and Med. Sci.*, 1946, **24**, 197.

TABLE I.

Substrate	Cu mm CO_2 evolved in 30 minutes: Cholinesterase from human A cells	Cholinesterase from human AB cells	Type I pneumococcus autolysate	Type V pneumococcus Autolysate	Type V pneumococcus Filtrate	Normal rabbit serum	Normal G.P. serum	Normal human serum
Tributyrin .03 M	0	0	38	43	14	200	256	184
Acetyl choline .003 M	40	34	—	—	—	—	—	—

inhibit the hemagglutinating action of vaccinia and ectromelia viruses but did not affect the agglutination of red cells by influenza virus.

The discovery[6] of an esterase in pneumococcus cultures, subsequently identified as cholinesterase[7] suggested to us that the latter enzyme might bear a causal relationship to the inhibition of influenzal hemagglutination. While some of our results do not fit smoothly into such an explanation, they are not altogether contradictory and the data obtained seem of sufficient importance to warrant publication.

Materials and Methods. Pneumococcal esterase was prepared from Type I and Type V cultures grown in a beef heart infusion hormone broth. In one method of preparation, after 18-24 hours incubation the cultures were adjusted to pH 7 with N/1 NaOH, were refrigerated overnight (for increased autolysis) and then centrifuged. The supernatant was Seitz-filtered. A second preparation was obtained by repeated freezing and thawing (6 cycles) of saline suspensions of once-washed 18-hour cultures. In the following discussion the first preparation is designated as pneumococcal filtrate, the second as pneumococcal autolysate.

For comparative purposes a partially purified specific cholinesterase was prepared from human red cells by the method of Mendel and Rudney.[8]

The inhibitory substances were titrated by a modification of the Hirst agglutinin-inhibition technic in which successive 2-fold dilutions of the inhibiting substance were substituted for serum dilutions. In all such tests 10 agglutinating units of PR8 influenza virus were employed per tube and a 0.75% chicken red-cell suspension was used instead of the heavier suspension recommended by Hirst. Naked-eye readings are facilitated by the use of the lighter cell suspensions. Inhibition titers are 2- to 4-fold higher than when 1.5% suspensions are used.

Experimental. Table I records the esterase activity of the above preparations and also of normal rabbit, guinea pig, and human sera. Schachter's modification[9] of the Ammon-Warburg technic was used with tributyrin as the substrate. Since the esterase of blood cells is a specific cholinesterase, acetyl choline was used in determining its activity.

In Table II are recorded the results of agglutinin-inhibition tests using normal human and animal sera as inhibiting agents as well as the esterases prepared from pneumococci and from human red cells.

To determine whether the effect of the pneumococcal inhibitory substance was due to action upon the virus, an unconcentrated allantoic fluid having a CCA titer of 1/1280 was mixed in equal proportions with Type

3 Friedewald, W., Miller, E. W., and Whatley, L. R., *Soc. Am. Bacteriologists, Abst. Proc.*, 47th meeting, 1947, 62.

4 Liebmann, A. J., Perlstein, D., and Snyder, G. A., *Soc. Am. Bacteriologists, Abst. Proc.*, 47th meeting, 1947, 63.

5 Woolley, D. W., and Green, R. H., *Soc. Am. Bacteriologists, Abst. Proc.*, 47th meeting, 1947, 63.

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8 Mendel, B., and Rudney, H., *Biochem J.*, 1943, **37**, 59.

9 Schachter, R. J., *Am. J. Physiol.*, 1945, **143**, 552.

TABLE II.

Source of inhibiting material	Type I pneumococcus filtrate	Type I pneumococcus autolysate	Type V pneumococcus filtrate	Type V pneumococcus autolysate	Cholinesterase from human A cells	Cholinesterase from human AB cells	Normal G.P. serum	Normal rabbit serum	Normal human serum (Group O)	Normal human serum (Group AB)
Agglutinin-inhibition titer	1/16	1/128	1/256	1/512	1/32	1/64	1/512	1/2048	1/4096	1/512

V pneumococcal filtrate diluted 1/5 and the mixture was incubated at room temperature for 1⅛ hours. The virus thus treated was then diluted 1/128 with physiological saline to make a final virus concentration of 10 agglutinating units per cc and a final dilution of the filtrate of 1/640 (beyond the concentration at which it could inhibit agglutination). When a red cell suspension was added typical agglutination occurred. Virus similarly treated with red-cell cholinesterase or with guinea pig serum was also still capable of agglutinating chicken red cells.

The pneumococcal filtrate, diluted 1/5 was then incubated at room temperature with an equal volume of 7.5% chicken red-cell suspension for 1½ hours after which the cells were washed twice, resuspended in 0.75% concentration and tested for agglutinability. They were found to be no longer agglutinated by PR8 virus. Cholinesterase from human red cells had no such effect upon the agglutinability of chicken red cells.

According to Avery and Cullen[6] pneumococcal esterase suffers progressive loss of activity at temperatures above 50° C and becomes completely inactive within 10 minutes at 70° C. McCrea has reported[10] similar progressive destruction between 56° C and 65° C of the substance in normal rabbit serum which inhibits influenzal hemagglutination and complete inactivation when such serum was heated for 30 minutes at any higher temperatures. Had we been able to confirm McCrea's observations, a striking analogy could have been established between the inhibiting agent and the cholinesterases studied. As Table III shows, however, the inhibitory power of normal guinea pig and rabbit sera were found to be either unaffected or were somewhat enhanced by temperatures between 60° C and 70° C. Type V pneumococcal filtrate and human red-cell cholinesterase, on the other hand, were entirely inactive after being heated to 60° C for half an hour.

It is interesting to note (Table IV) that when the inhibition test is performed at room temperature agglutination may occur at first

[10] McCrea, J. F., *Australian J. Exp. Biol and Med. Sci.*, 1946, **24**, 283.

in tubes in which with further incubation the clumps will be dispersed under the influence of the inhibiting agent. Whether the agent is a serum or a pneumococcal preparation, it appears to be slower in its action at this temperature than the agglutinating factor but in time is capable within its effective concentration of counteracting it completely.

Discussion. Tables I to IV, inclusive, reveal significant similarities in the action of pneumococcal filtrates, human red-cell extracts, and sera of 3 species of non-immunized animals. Since chicken red cells exposed to the Type V pneumococcal preparation rendered them refractory to agglutination by PR8 virus whereas the human red-cell extract failed to produce any such effect, some doubt is aroused regarding the relationship between specific cholinesterase (which the human cell extract contains) and the inhibitory phenomenon.

When these discordant findings have been considered, however, the fact remains that all of the agents employed in this study were capable of inhibiting influenzal haemagglutination and all contained either specific or non-specific cholinesterase.

Filtrates of a number of other organisms were tested for the inhibitory effect, but all were found to be inactive. The organisms tried included *Staph. aureus, Strept pyogenes, Strept. mitior, C. diphtheriae, C. hoffmanni, E. coli, B. subtilis,* and *Prot. vulgaris.* All of these organisms have been reported[11] to contain no cholinesterase.

TABLE III.
Agglutination-inhibition Titers.

Inhibiting agents	Treatment			
	Unheated	60°C ½ hr	65°C* ½ hr	70°C* ½ hr
Type V pneumococcus filtrate	1/256	<1/4		
Red-cell cholinesterase	1/32	<1/4		
Guinea pig serum	1/320	1/1280	1/1280	1/1280
Rabbit serum	1/320	1/320	1/1280	1/2560

* Sera were diluted 1/10 with physiological saline prior to heating.

Summary. 1. Filtrates and autolysates of Type I and Type V pneumococcus cultures inhibited the agglutination of chicken red cells by influenza virus. 2. Chicken red cells treated with such a filtrate (or autolysate) could not then be agglutinated by the virus. 3. Human red-cell extracts and normal sera of man, guinea pigs, and rabbits were also shown to be capable of inhibiting agglutination of chicken red cells by influenza virus, although some differences of behavior were noted. 4. The presence of a cholinesterase in each of these agents was shown and their possible significance in agglutinin inhibition was discussed.

[11] Vincent, D., and de Trat, J., *Compt. Rend. Soc. Biol.*, 1945, **139**, 1148.

TABLE IV.
Inhibition Titers.

Incubation time	G.P. serum vs PR8 (Type A)	G.P. serum vs Lee (Type B)	Rabbit serum vs PR8 (Type A)	Rabbit serum vs Lee (Type B)	Type V pneumo filtrate vs PR8
25 min	<1/10	1/10	0	1/20	1/10
90 min	1/1280	1/2560	1/640	1/80	1/80
16 hr*	1/2560	1/2560	1/1280	1/1280	1/160

* Placed in 4°C refrigerator after 90 minutes at indicated temperature.

15970 P

Ovarian Cholesterol Levels During the Reproductive Cycle of the Rat.*

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Evidence that ovarian cholesterol may be a precursor of the ovarian hormones, particularly progesterone, has been by inference, both positive and negative. Comparison of results from previous studies on cholesterol variations during the ovarian cycle is difficult because of the lack of uniformity in presenting data, *i.e.*, some report the free cholesterol and others only the cholesterol esters. By histochemical methods, no changes were observed in ovarian cholesterol during the reproductive cycle in humans[1] although chemical analysis of human corpora lutea indicated a significant decrease in cholesterol esters only in the actively functioning corpus luteum of pregnancy.[2] In the pig, the cholesterol ester content of the corpora lutea was found to vary inversely with the activity of the gland.[3] Boyd[4] found that in the whole ovary of the rabbit, there was an increase in the free cholesterol during the first half of pregnancy followed by a decline while the cholesterol esters increased only during the last half of pregnancy. These results were interpreted as indicating that the rabbit corpora lutea reach a peak of activity about the middle of gestation. No change in free cholesterol was observed in the ovaries of guinea pigs during the course of gestation.[5] Based on histochemical studies of the corpora lutea of diestrum in the rat, Everett[6] strongly suggested that cholesterol serves as a precursor of progesterone.

A systematic examination of cholesterol in the ovary of the rat has been undertaken in this laboratory, and this report will show the changes in cholesterol level of the ovary in diestrum, pseudopregnancy, and at several intervals during pregnancy and lactation.

Methods. Total cholesterol was determined by the method of Chamberlain[7] which consists essentially in saponification of the lipids in alkaline alcohol, extraction of the lipids with ether, and determination of the cholesterol directly on the washed ether-extract residue by the Liebermann-Burchard reaction. A Bausch and Lomb colorimeter was used for color comparison. This procedure gave results which were comparable with those obtained by the digitonin precipitation method[8] both in recoveries of pure cholesterol from solution as well as from pooled ovarian tissue. For example, the average value of 3 determinations on an ovarian tissue sample yielded 0.39 mg by the digitonin method as compared to 0.37 mg for aliquot samples using the Chamberlain method.

The stages of the reproductive cycle at which the ovaries were analyzed are given in the table. Pseudopregnancy was induced by electrical stimulation of the cervix. The ovaries were trimmed of extraneous tissue under the binocular dissecting microscope and weighed to the nearest 0.1 mg.

Results. The cholesterol values are best compared on the basis of parts per thousand. It is noted in Table I that the highest levels were found on the 8th day of pseudopregnancy, 10th day of pregnancy and the 10th day of lactation. The cholesterol in the ovaries on the 15th day of lactation, while not as high as obtained above, nevertheless was significantly higher than that found at diestrum (P value between .05 - .02). The highest levels of cholesterol were found only at a time when it

* Aided by a grant from the Sage Fund provided by the Cornell University Trustee-Faculty Committee on Research.

† Fellow of the Schering Corporation.

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5 Boyd, E. M., *J. Biol. Chem.*, 1936, **112**, 591.

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8 Kelsey, F. E., *J. Biol. Chem.*, 1939, **127**, 15.

TABLE I.
Cholesterol in Rat Ovaries During the Reproductive Cycle.*

	Period (days)	No. of rats	Body wt g	Ovarian wt mg	Total cholesterol mg	Cholesterol parts/1000	No. of fetuses or suckling young
Control	diestr.	10	223	62.5 (36.3- 94.8)	.45 (.30-.65)	7.3 (5.0- 9.0)	—
Pregnancy	10	7	223	60.9 (40.5- 66.9)	.64 (.53-.74)	10.6 (8.6-13.1)	7
	14	9	259	83.5 (73.5- 99.8)	.63 (.44-.79)	7.6 (4.8- 9.8)	9
	18	5	331	104.8 (100.0-105.2)	.80 (.72-.93)	7.7 (7.0- 9.3)	12
Pseudopreg.	8	6	218	43.5 (35.8- 53.8)	.47 (.36-.58)	11.0 (7.7-13.7)	—
Lactation	1	5	224	67.1 (60.0- 77.2)	.49 (.46-.51)	7.3 (6.4- 8.5)	9
	5	6	223	58.4 (41.4- 78.7)	.41 (.30-.51)	7.2 (5.1- 9.7)	8
	10	7	220	49.9 (42.3- 58.2)	.54 (.36-.68)	11.4 (6.2-14.3)	8
	15	7	245	52.0 (34.6- 71.3)	.44 (.30-.52)	8.6 (7.7- 9.7)	8

* All values are average, the range indicated by ( ).

is possible to demonstrate placentomata in the uterus and thus when there must be large amounts of circulating progesterone.[9] It is possible to demonstrate placentomata in the uterus on the 15th day of lactation provided an adequate number of young are suckling (8 or more).

The total ovarian cholesterol was found to be highest on the 18th day of pregnancy yet it is impossible to secure placentomata in the non-pregnant horn of a unilateral pregnant rat at this time.[9] Since functional corpora lutea or progesterone in the rat are necessary for the maintenance of pregnancy, it seemed odd that the cholesterol content of the ovaries in parts per thousand did not remain high throughout pregnancy. This suggests further studies on the ovaries of pregnant rats. It cannot be stated conclusively from these results that cholesterol acts a precursor of progesterone but in certain instances it appears that high ovarian cholesterol is associated with high levels of progesterone secretion.

[9] Burrows, H., *Biological Actions of Sex Hormones*, Cambridge Univ. Press, 1945, 419.

15971

Turnover of Serum Protein in Adrenalectomized Rats.*

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In previous experiments adrenal cortical activity was found to have no effect on the concentration of serum antibodies or on any other fraction of the serum proteins.[1] This finding, however, did not preclude the pos-

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We wish to express our thanks to Dr. David Shemin for providing the tagged glycine used in these experiments and for determining the N^{15} values.

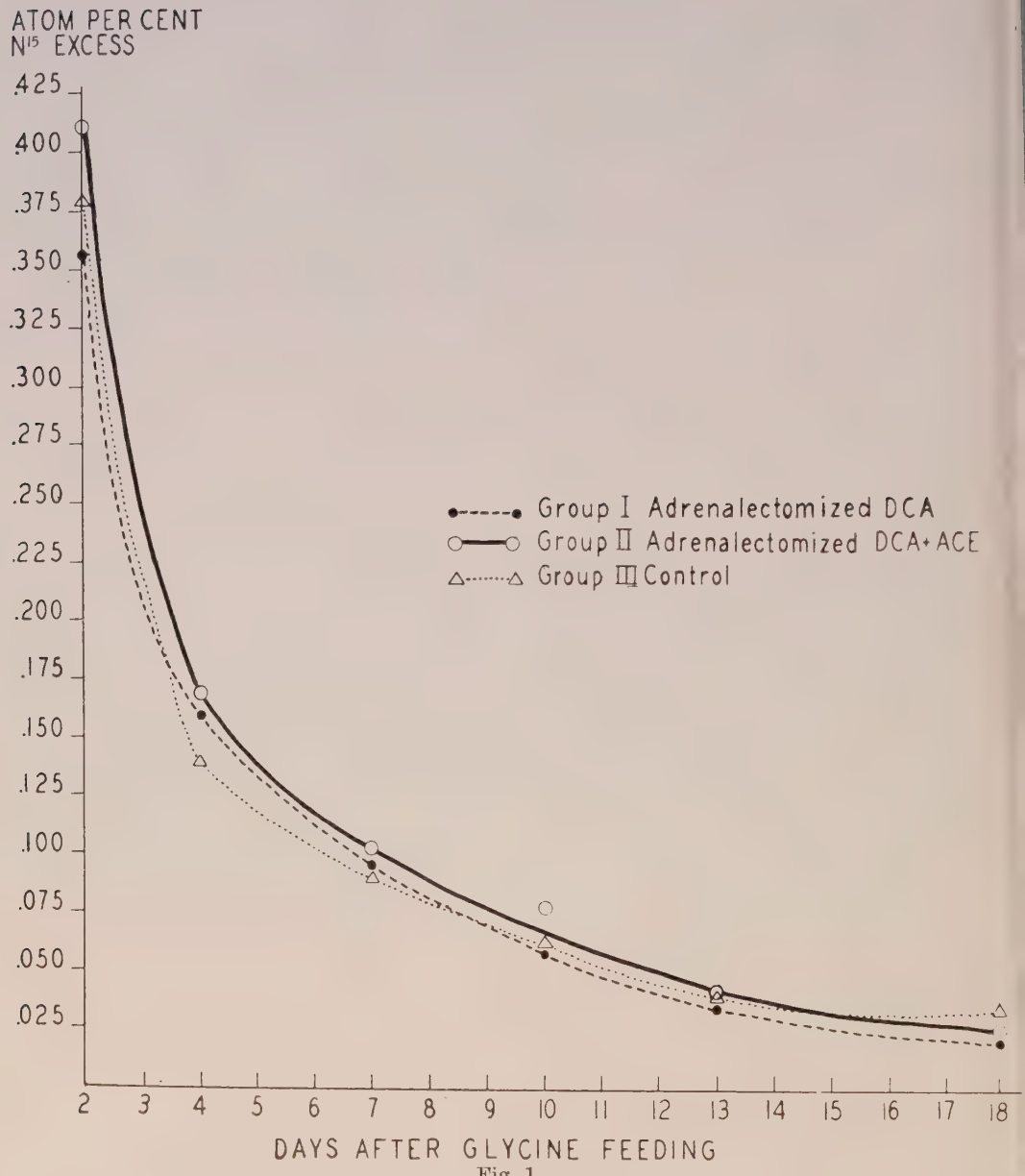

Fig. 1.

The half lifetime of serum proteins, estimated from the rate of decline of atom % N^{15} excess, is the same in all 3 groups.

sibility that the adrenal cortical hormones exerted a considerable influence on the turnover of the serum proteins. It therefore seemed desirable to determine the rates of synthesis and degradation of the serum proteins in adrenalectomized rats, particularly in view of the widely held belief that adrenal cortical activity influences protein catabolism.[2]

Methods and materials. Nine adult male

[1] Eisen, H. N., Mayer, N. M., Moore, D. H., Tarr, R. R., and Stoerk, H. C., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 301.

[2] Long, C. N. H., Katzin, B., and Fry, E. G., *Endocrinology,* 1940, **26**, 309.

TABLE I.
Rate of Decline of Atom % N^{15} Excess in Total Serum Proteins.

Group	Procedure	Animal No.	Days after conclusion of feeding isotopically-labelled glycine					
			2	4	7	10	13	18
I	Adrenalectomy	58	—	.162	.093	.059	.038	.018
	DCA	61	.345	.155	.091	.052	.030	.013
		67	.368	.167	.105	.064	.038	.033
II	Adrenalectomy	68	.343	.200	.134	.068	.051	.033
	DCA	73	.473	.159	.092	.065	.044	.024
	ACE	65	.420	.151	.105	.084	.034	.017
III	Controls	74	.399	.142	.107	.072	.049	.033
		75	.353	—	.083	.065	.038	.031
		69	.381	.145	.081	.049	.038	.029

albino rats of the Sherman strain, ranging in weight from 250 to 295 g were divided into 3 groups. The animals of Group I were bilaterally adrenalectomized under ether anaesthesia and were maintained during the post-operative period on Rockland rat diet, subcutaneous injections of desoxycorticosterone acetate (DCA),§ 0.4 mg on alternate days, and on drinking water containing 1% sodium chloride. The animals of Group II were treated similarly. However, from the ninth post-operative day to the end of the experiment they received, in addition, daily injections of 0.5 ml adrenal cortical lipoextract (ACE).‖ Group III was composed of unoperated, uninjected control animals.

From the tenth to the twelfth post-operative days animals in all three groups were fed glycine tagged with heavy nitrogen. The isotopically labelled glycine was incorporated into the diet and administered so that each animal received 0.4 mg per gram body weight. Beginning 48 hours after the cessation of the glycine feeding, and repeated at intervals of from 2 to 10 days, 0.5 ml of blood was collected from a freshly cut surface of the tail and the total serum proteins were examined for their content of N^{15} by methods previously described[3]. After 30 days the animals were exsanguinated and their blood analyzed for total protein, albumin, globulin, and total non-protein nitrogen.¶

Results. The animals of all 3 groups gained approximately 10% in body weight. The serum total proteins, albumin, globulin, and non-protein nitrogen values determined at the end of the experiment (40th post-operative day) were the same in Group I and in Group II and were within normal limits. The heavy nitrogen contents of the serum proteins are given in Table I and in Figure 1.

Discussion. Following the feeding of labelled glycine, the N^{15} which had been incorporated into the serum proteins was replaced by N^{14} at a rate which was identical for all 3 groups. These data indicate that the rate of synthesis of serum proteins is not affected by adrenalectomy or by the administration of ACE to adrenalectomized rats. Since the total "pool" of serum proteins seems to have been approximately the same in the several groups, the rate of serum protein breakdown appears likewise to have been no different. These data do not exclude the possibility that adrenal cortical activity exerts an influence on the turnover of *tissue* proteins.

The half lifetime of serum proteins in the rat (1½ days) was found to be considerably briefer than the value (about 12 days) found in rabbits by Schoenheimer et al.[4] Unless the

§ Generously provided by Schering Corporation, Bloomfield, N. J.

‖ Upjohn Company, Kalamazoo, Mich.

[3] Rittenberg, D., Keston, A. S., Rosebury, F., and Schoenheimer, R., *J. Biol. Chem.*, 1939, **127**, 291.

¶ We wish to thank Miss Genevive Corbett for these determinations.

[4] Schoenheimer, R., Ratner, S., Rittenberg, D., and Heidelberger, M., *J. Biol. Chem.*, 1942, **144**, 545.

repeated bleedings exerted a more profound effect on rats than on rabbits, no explanation for this difference is apparent.

Summary. The turnover of serum protein in the rat is unaffected by adrenalectomy, or by the administration of adrenal cortical lipo-extract (ACE) to adrenalectomized animals.

15972

Increased Dental Caries Activity in the Syrian Hamster Following Desalivation.*

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The Syrian hamster (*Cricetus auratus*) has been used in the study of experimental dental caries and appears well suited for such investigation since the disease process in this animal appears similar to that described in humans.[1] Furthermore, the administration of fluorine with a caries producing diet has been shown to reduce the caries incidence,[2] a finding which roughly parallels observations in man.[3] It appeared of some importance to determine whether removal of the major salivary glands would be accompanied by increased caries activity since it is well known that removal, agenesis, or greatly reduced function of the salivary glands in humans is accompanied by rampant decay.[4-5] Such a response might be expected since increased caries activity subsequent to salivary gland extirpation has been observed in the albino rat.[6-9]

Experimental. The animals were of pure inbred stock and were raised on Purina Rabbit Checkers plus weekly supplements of fresh vegetables. They were kept in metal cages with wood shavings for bedding. When 42 days old (± 4 days), the animals were separated into a control group (Group I) and an experimental group (Group II). A comparable supplementary group (Group Ia) was available for additional comparisons. The experimental animals were desalivated during a period of 2 weeks. The parotid, submaxillary, and major sublingual glands were removed, under ether anesthesia, through a single midline incision beginning about 5mm from the mandibular symphysis and extending to the manubrium. Control animals were not operated upon. One week after the last operation,‡ an experimental diet and tap water were provided *ad libitum* to Groups I and II. The diet consisted of whole wheat flour, 40%; whole powdered milk, 30%; glucose, C.P., 20%; alfalfa, 5% brewers yeast, 4%; and sodium chloride, 1%. The supplementary control animals (Group Ia), which were used for another study, were of the same age but had been started on the diet 25 days previously.

Animals were weighed at weekly intervals.

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‡ Four animals failed to survive the operation and, consequently, have not been included in the data.

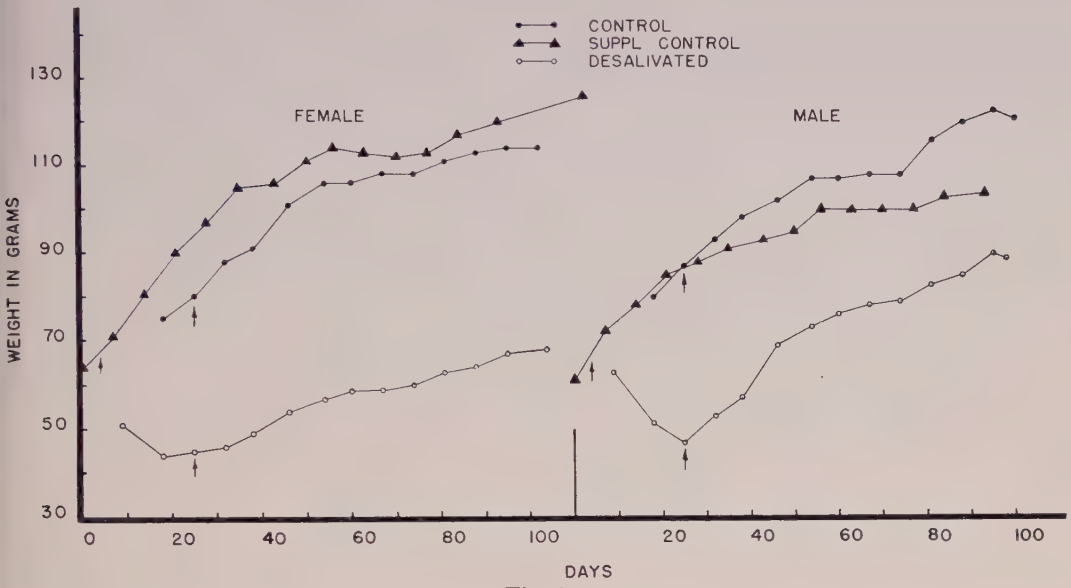

Fig. 1.
Average weight curves for the 3 groups. Arrows indicate the time at which the experimental diet was begun.

Group I and II animals were sacrificed over a 6 day period starting 73 days after the experimental diet had begun. An autopsy was done on each of these animals. Male animals in the supplementary group were killed after 93 days on the diet and female animals after 111 days. Heads were preserved in 10% formaldehyde, stripped of soft tissue, and scored;[10] the number of carious teeth (M. A.), the number of cavities (C. N.), and the total score (T. S.) were recorded. Total score (T. S.) is an arbitrary figure which represents the amount of tooth structure destroyed. All teeth were scored by the same investigator. Data independently obtained for Group Ia animals by the co-author were in excellent agreement.

Differences among the means of average M. A., average C. N., and average T. S. for each of the groups compared were analyzed by the Student-t method.[11] Comparison was made between Group Ia♂♂ and ♀♀; Group Ia and I ♂♂; Group Ia and I ♀♀; Group I and II ♂♂; and Group I and II♀♀. An arbitrary level of significance of $p = .01$ was chosen; all results less than this value were considered significant.

Differences among the groups were further compared by the calculation of "percentage of molars affected" and "average percentage of tooth destroyed" for each of the several types of molars. This method of calculation is illustrated beneath Table III.

Results. Average weight curves for the 3 groups are presented in Fig. 1. Desalivated animals lost weight following the operation and remained lighter than those of the control groups. Desalivated females were especially uniform in their weight response. Control animals were sleek, fat, and healthy in appearance. The experimental animals were noticeably emaciated, were more active, and showed at autopsy, a pronounced reduction in depot fat.

Inspection of the mouths prior to administration of the experimental diet disclosed a generalized dryness of the oral tissues in the case of the operated animals whereas the mucous membranes of Group I animals were bathed by copious amounts of salivary secretion.

Caries scores and statistical findings are presented in Tables I and II. *Control groups.* Despite the fact that Group Ia males had been maintained on the experimental diet

[10] Keyes, P. H., *J. D. Res.*, 1944, **23**, 439.

[11] Fisher, R. A., *Statistical Methods for Research Workers*, 7th Ed., 1938, Chapt. 5.

TABLE I.
Effect of Desalivation on Caries Activity.

Group	Male				Female			
	Animal No.	Max. + Mand. scores M.A.	C.N.	T.S.	Animal No.	Max. + Mand. scores M.A.	C.N.	T.S.
Suppl. control	27	9	11	18	42	1	1	¼
	82	9	10	31	12	3	3	1
	96	5	5	34	15	6	8	5
	69	10	12	59	57	5	6	7
	89	12	14	77	34	6	6	8
	32	11	11	104	75	6	6	9¼
	104	12	14	107	98	6	7	15
	51	12	14	132	122	8	9	29
					50	9	11	64
Avg		10	11	70		6	6	16
Control	123[1]	5	5	2¼	71[3]	1	1	½
	112[2]	6	6	11	46	4	5	2
	124	12	12	73	100	8	8	13
	13	11	15	111	88[4]	4	4	13
					63[5]	7	8	29
Avg		9	10	49		5	5	12
Experimental	122×[1]	11	15	72	68[3]	12	12	208
	113[2]	12	14	166	×	12	13	239
	92	12	15	221	93[4]	12	12	257
					64[5]	12	12	278
Avg		12	15	153		12	12	246

M.A. = Molars affected.
C.N. = Cavity number.
T.S. = Total score.
Total scores of 138 and 144 indicate complete destruction of maxillary and mandibular molar teeth, respectively.
Animals indicated by same superscript are litter mates.

for 18 days less than the females, the average M. A., C. N., and T. S. were higher for male animals than for female. This difference was statistically significant. Differences between Groups Ia and I were not significant. *Experimental group.* The incidence of dental caries in both male and female animals was increased by desalivation. Average values for M. A., C. N., and T. S. are in every case higher for desalivated animals than for controls. Differences among the male hamsters (Groups I and II) were not statistically significant; differences among famales of the same groups were highly significant. Several factors may have obscured statistical differences among the male animals; (1) high caries incidence among unoperated animals, (2) greater variability of response in the male and (3) the small number of animals employed. It is interesting to note that although male animals ordinarily accumulate more dental decay, females experience a greater amount of tooth destruction subsequent to desalivation.

Considerable variation was observed among individual molar types with respect to percentage of teeth decayed and average percentage of tooth destroyed (Table III). However, in every case but one (♂, Group II, mand. 3rd molars) both measures of caries activity were greater for the desalivated group than for either control group. Every tooth of the experimental animals with one exception (as cited above) showed evidence of dental caries, while in several instances complete or almost complete destruction of all crowns was recorded, e. g. ♀, Group II, max. molars.

Discussion. Under the conditions of this experiment, desalivation of hamsters was followed by a definite reduction in body weight especially in the female animals. Decreased food consumption may have contributed to this

TABLE II.
Statistical Evaluation of Caries Scores.

Comparison	Avg	t	p
Group Ia ♂♂ vs. ♀♀	M.A.	3.80	.002
	C.N.	3.50	.004
	T.S.	3.40	.004
♂♂ " " vs. Group I	M.A.	0.77	.45
	C.N.	0.73	.50
	T.S.	0.70	.50
♀♀ " " vs. " I	M.A.	0.55	.60
	C.N.	0.67	.50
	T.S.	0.46	.65
♂♂ " I vs. " II	M.A.	1.73	.14
	C.N.	2.10	.09
	T.S.	2.05	.10
♀♀ " I vs. " II	M.A.	5.90	.0006
	C.N.	5.27	.001
	T.S.	14.9	<.0002

$$t = \frac{m_1 - m_2}{\sqrt{\sigma^2_{m_1} + \sigma^2_{m_2}}}$$

where m_1 and m_2 = means compared

σ_{m_1} and σ_{m_2} = unbiased estimate of the standard deviation of the means

$$\sigma_m = \frac{\sigma}{\sqrt{N}}$$

σ = unbiased estimate of standard deviation

N = No. of animals

$(N_1 + N_2 - 2)$ = Degrees of freedom in t

TABLE III.
Caries Incidence as Found in Individual Molars.

	Molar teeth	Group Ia (Suppl. control) % molars affected	Group Ia (Suppl. control) Avg % of tooth decayed	Group I (Control) % molars affected	Group I (Control) Avg % of tooth decayed	Group II (Experimental) % molars affected	Group II (Experimental) Avg % of tooth decayed
	Female						
Max.	1st	60.	1.6	40.	0.56	100.	97.
	2nd	40.	3.0	50.	8.4	100.	100.
	3rd	30.	2.2	00.	0.0	100.	100.
Mand.	1	70.	7.0	80.	2.3	100.	93.
	2	60.	17.	60.	12.	100.	90.
	3	30.	1.3	10.	0.10	100.	55.
	Male						
Max.	1	90.	7.8	80.	2.0	100.	53.
	2	70.	21.	40.	13.	100.	61.
	3	80.	28.	80.	14.	100.	54.
Mand.	1	90.	40.	90.	16.	100.	68.
	2	90.	34.	100.	49.	100.	75.
	3	80.	21.	50.	15.	80.	13.

Sample calculation (Maxillary 1st molar, Group Ia, female):

Percentage of molars affected = $\frac{\text{No. of carious max. 1st molars}}{\text{Total No. of max. 1st molars}} \times 100 = \frac{11}{18} \times 100 = 60.\%$

Avg percentage of tooth destroyed = $\frac{\text{Sum of total scores of all carious max. 1st molars}}{\text{Sum of available total scores of all max. 1st molars}} \times 100 = \frac{7.75}{486} \times 100 = 1.6\%$

effect for several measurements on group food intakes revealed a consistent reduction in the desalivated group. The effect of the operation, per se, cannot be stated since a sham operation was not performed on either of the control groups. Weight loss following operation has been observed in the rat.[12] Reports on the subsequent changes in weight after recovery of rats from comparable operations are not in agreement. Hukusima[7] found a reduction in body weight; data presented by Cheyne[12] showed an appreciable reduction in 5 of the 8 groups studied although the animals remained in good health. Weisberger et al.[9] reported no apparent ill effects on subsequent growth and development.

The higher incidence of decay observed in male hamsters (Group Ia) is in agreement with previous reports.[1,13–16]

Since Group Ia was on the experimental regimen for appreciably longer periods of time than Group I, a higher incidence of decay would be expected in the former. This proved to be true although the differences were not large and were not shown to be significant. This finding tended to increase the number of control observations and strengthened the argument that changes observed in desalivated animals could not be explained on the basis of normal variation.

The increased caries incidence following salivary gland extirpation in the hamster conforms with observations made in the rat and in man. It is difficult to compare the response of the rat and hamster because of variable experimental conditions and dissimilar scoring technics. In the rat, there is a rise in number of animals[6–7] and number of teeth[8] affected as well as an increase in the number of carious lesions.[8] With the hamster, large differences are noted only in the amount of tooth destruction, especially in the female. The rapidity of the carious process may have obscured an increase in number of cavities formed.

The mechanism of increased caries activity cannot be explained by the present findings. Two possibilities suggest themselves: alteration in systemic factors and/or alteration in local factors. The latter explanation is probably more correct. Interference with salivary flow (as observed) would reduce the normal cleansing action of the saliva. In addition, it is not unlikely that the saliva of these rodents contains antibacterial agents and other compounds similar to those observed in human saliva, which are known to inhibit the growth[17] or oppose the action of oral bacteria.[18–19]

Summary and conclusions. 1. Seven hamsters (3 ♂♂, 4 ♀♀) were desalivated and compared with a control group (4 ♂♂, 5 ♀♀) and a supplementary control group (8 ♂♂, 9 ♀♀). It has been found that the animal can be desalivated easily and that it will usually survive operations.

2. Under the experimental conditions, extirpation of the glands resulted in a sharp reduction in body weight. Subsequently, body weight remained below that of the control groups.

3. Dental caries activity following operation was increased in both male and female hamsters although the difference was not shown to be statistically significant in the case of male animals. It is probable that a comparable response does occur in males but that a larger group of animals would be necessary for an unequivocal demonstration. Response following desalivation was similar to that reported previously for albino rats and humans.

4. Unoperated male animals experienced more dental decay than did unoperated female animals.

The authors are indebted to Dr. James A. Rafferty, Department of Pathology, for statistical evaluation of the data and to Dr. Harold C. Hodge, Department of Pharmacology and Toxicology, for helpful criticism.

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15973

Observations on the Conversion of Prothrombin to Thrombin.

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Evidence from several sources has indicated that the rate of formation of thrombin in plasma upon the addition of a given thromboplastin is affected by some factor besides the concentration of the prothrombin itself. Seegers, Loomis and Vandenbelt[1] found that thrombin was formed more slowly from very highly purified prothrombin than from prothrombin naturally contained in plasma. In studies of patients who had cirrhosis[2] or pernicious anemia,[3] of newborn infants[4] and of normal dogs and rabbits,[5] the concentration of prothrombin in the plasma has been reported to be less when determined by the 2-stage procedure of Warner, Brinkhous and Smith[5] than by the one-stage method of Quick.[6] In Quick's method the time required for conversion of prothrombin to thrombin constitutes part of the observed clotting time. These differences are attributed by the originators of the 2-stage method to an increased rate of conversion of prothrombin in the one-stage technic. On the other hand Quick[6] thought the differences were due to incomplete conversion of prothrombin in the high dilution required by the 2-stage method. However, when the plasma of patients treated with dicumarol[7] or normal plasma after storage[8] are studied by both methods the 2-stage procedure gives the higher values. Quick[9] has presented evidence that the increase in prothrombin time of plasma after storage is due to the disappearance of a substance termed "prothrombin A" which remains in fresh plasma depleted of prothrombin by treatment of patients or animals with dicumarol or addition of aluminum hydroxide to the plasma. Fantl and Nance[10] also found that plasma freed of prothrombin by treatment with barium carbonate still contained a substance which accelerated the conversion of prothrombin to thrombin. Because in blood coagulation the rate of thrombin formation is more important than the total amount of available prothrombin, such a factor or factors affecting conversion appear to deserve further study.

Method. The prothrombin time of fresh or stored (one to 2 months) human plasma was determined by Quick's method as previously described,[11] except that just before addition of thromboplastin, 0.1 ml of a 0.9% solution of sodium chloride, or whatever material was being tested for action on the conversion rate, was added. Thus, the volume of the clotting system was increased from 0.3 to 0.4 ml.

Standard thromboplastin was prepared by Quick's original method. With this preparation, normal plasma has a prothrombin time of from 17 to 19 seconds. Fresh tissue thromboplastin was made by macerating fresh or frozen rabbit brain with twice its weight of ice-cold normal saline solution. A commercial preparation of acetone-dehydrated rabbit brain* was used. Thromboplastin was prepared from human placenta by the method

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4 Owen, C., Hoffman, G., Ziffren, S., and Smith, H., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 181.

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6 Quick, A., *The Hemorrhagic Diseases and the Physiology of Hemostasis,* Springfield, Ill., Charles C Thomas, 1942, pp. 36 and 40.

7 Hurn, M., Barker, N., and Mann, F., *Am. J. Clin. Path.*, 1947, **17**, 712.

8 Lord, J., and Pastore, J., *J. A. M. A.*, 1939, **113**, 2231.

9 Quick, A., *Am. J. Physiol.*, 1943, **140**, 212.

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11 Hurn, M., Barker, N., and Magath, T., *J. Lab. and Clin. Med.*, 1945, **30**, 432.

of Zondek and Finkelstein.[12]

The materials tested for prothrombin-converting activity were plasma and serum from human beings and crude platelet extract. The platelet extract was prepared as follows. Oxalated plasma was separated from the cells by gentle centrifugation; then the suspension of platelets thus obtained was centrifuged for 30 minutes at 3,000 revolutions per minute. The plasma was carefully drained from the tubes, after which the sticky pellet of sediment was removed with a stirring rod. The sediment, which thus contained a minimal amount of plasma, was triturated in a mortar with 0.9% solution of sodium chloride containing 0.001 molar sodium oxalate. One milliliter of this fluid was used for the amount of sediment obtained from 16 ml of plasma. Serum was used only after it had stood 3 to 4 hours at room temperature to allow for destruction of thrombin. Neither the platelet extracts nor the serum used caused clotting when mixed with oxalated plasma. The plasma tested was drawn and oxalated as for a prothrombin determination or in some experiments the silicone technic of Jaques and his associates[13] was used. When the silicone technic was used all pipets and tubes used in handling the plasma throughout the procedure were coated with silicone.

Results. Fresh plasma, serum and platelet extracts were all found to reduce the prothrombin time of stored plasma markedly (Tables I, II and III). The fresh plasma was so highly diluted that little of the effect could be attributed to its content of prothrombin; the same was true of platelet extract. Platelet extract showed considerably more prothrombin-converting activity than did serum or plasma. When the thromboplastin of rabbit brain was replaced with normal saline solution the clotting time was greatly prolonged, showing that the platelet extract potentiated the action of the tissue thromboplastin. Platelet extract was observed to potentiate the action of thromboplastin from dried rabbit brain, fresh rabbit brain, acetone-dehydrated rabbit brain and human placenta on stored

TABLE I.
Effect on Stored Plasma with Standard Thromboplastin of Plasma and Serum Drawn from Same Subject at the Same Time.

Material added to plasma	Dilution	Clotting time, sec
Saline		82
Plasma	1:10	36
	1:20	41
	1:40	49
	1:80	57
Serum*	1:10	31
	1:20	37
	1:40	43
	1:80	57

* Undiluted serum usually restored the clotting time to the normal value of 18 seconds.

TABLE II.
Potentiation of Standard Thromboplastin with Platelet Extract.

Plasma	Material added	Thrombo-plastin	Clotting time, sec
Stored	Saline	Standard	90
	Platelet extract	"	13
	" "	None	86
	Plasma from which extr. made dil. 1:5	Standard	28
Fresh	Saline	"	18
	Platelet extr.	"	13
	" "	None	47
	Saline	Standard dil. 1:50	39
	Platelet extr.	Standard dil. 1:50	23

TABLE III.
Potentiation of Fresh Tissue Thromboplastin with Platelet Extract.

Plasma	Material added	Thrombo-plastin	Clotting time, sec
Stored	Saline	Fresh tissue	52
	Platelet extr.	" "	15
	" "	None	45
Fresh	Saline	Fresh tissue	20
	Platelet extr.	" "	16
	" "	None	56
	Saline	Fresh tissue, dil. 1:50	32
	Platelet extr.	Fresh tissue, dil. 1:50	23

* Obtained from the Difco Laboratories, Inc., Detroit, Mich.

12 Zondek, Bernhard, and Finkelstein, Michael, PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 374.

13 Jaques, L., Fidlar, E., Feldsted, E., and Macdonald, A., *Canad. M. A. J.*, 1946, **55**, 26.

TABLE IV.
Effect of Plasma Drawn from Same Subject by Various Technics. Standard Thromboplastin. Stored Plasma.

Material added	Dilution	Clotting time, sec
Saline		145
Oxalated plasma	1:10	30
	1:40	49
Oxalated plasma (silicone technic)	1:10	31
	1:40	46
Native plasma* (silicone technic)	1:10	23
	1:40	36

* Tested within 5 minutes after blood drawn; blood clotted in original tube after 30 minutes.

plasma. A similar, but less striking, potentiation between platelet extract and tissue thromboplastin was observed when fresh plasma was used. Thus although the prothrombin-converting activity of fresh plasma is rather great it is still not maximal, especially if dilute thromboplastin is used. However, since the extracts represent 16 times their volume of plasma, it would seem reasonable that the activity of the plasma might not be less than that of its platelets. Plasma had the same converting activity when drawn and handled with silicone technic as when exposed to glass (Table IV). Thus it seems unlikely that this activity is a product of processes incident to the drawing of blood. Native plasma drawn with silicone technic appears to have more prothrombin-converting activity than the same plasma after decalcification. As a rule, all materials tested lost about 90% of their prothrombin-converting activity on standing 24 hours in the icebox. By heating at 56°C for 30 minutes, most of this activity was destroyed.

Comment. No specific term will be applied at present to the substance which potentiates the conversion of prothrombin to thrombin by tissue thromboplastin. This material appears to be the same as Quick's prothrombin A but it is not converted into thrombin since it remains in the serum. By definition, the conversion-favoring substance might reasonably be regarded as part of the thromboplastin complex. Quick[14] has justly emphasized the fact that if any appreciable amount of active thromboplastin were present in the circulation the blood would not be fluid. This material does appear to exist in the circulation and presumably cannot alone bring about formation of thrombin. The prothrombin-converting activity of the plasma, however, may well be a determining factor when the blood is exposed to a minimal amount of tissue thromboplastin, as it probably often is in cases of thrombosis.

Summary. Fresh plasma, serum and platelet extracts contain a material which potentiates the action of tissue thromboplastin on stored plasma. Platelet extract apparently contains a greater degree of this activity than plasma or serum and potentiates the action of tissue thromboplastin on fresh plasma. This factor favoring prothrombin conversion apparently is present in the circulating blood.

14 Quick, A., *The Hemorrhagic Diseases and the Physiology of Hemostasis,* Springfield, Ill., Charles C Thomas, 1942, p. 70.

15974

Chick Growth Factor in Cow Manure. VI. Effect on Hatchability and Storage in Hens.

MAX RUBIN, A. C. GROSCHKE, AND H. R. BIRD. (Introduced by T. C. Byerly.)

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Whitson *et al.*[1] showed that as the level of soybean oil meal in the diet of hens increased from 0 to 40% in increments of 10%, the hatchability of fertile eggs decreased in spite of apparently adequate quantities of the dietary factors known to affect hatchability. There was no other adverse effect. Whitson *et al.*[2] also showed that there was considerable improvement in hatchability when 8% of dried cow manure was included in a diet containing 30% soybean oil meal. Shortly thereafter, Bird *et al.*[3] reported that the inclusion of 6% cow (or steer) manure, 10% sardine meal, or 10% dried skimmilk in this diet corrected the detrimental effect on hatchability.

Rubin and Bird[4] prepared concentrates of a factor found in cow manure, which greatly stimulated the growth of chickens fed a diet containing 35 percent of soybean oil meal but no animal protein. It seemed likely that both growth and hatchability were influenced by the same unknown dietary factor in cow manure. To obtain further evidence on this point one of the concentrates which stimulated growth was tested for its effect on hatchability, and the results are reported herewith.

The hens used in part A of this experiment were Rhode Island Reds that were 10 months old when the experiment commenced. As chicks, they were fed diets which contained 2.5 to 4% of fish meal and they had access to grass range until they were 5 months old. During the latter half of the pre-experimental period they were fed diet 311 (Table I).

TABLE I.
Experimental Diets.

Ingredients	Diet 311 %	Diet 312 %
Yellow corn	57.0	78.3
Alfalfa leaf meal	5.0	5.0
Soybean oil meal	30.0	
Sardine fish-meal		10.0
Steamed bone meal	4.2	3.2
Limestone	2.3	2.0
Butyl fermentation solubles (250 μg riboflavin per g)	0.5	0.5
Salt (94% NaCl; 6% $MnSO_4$)	0.5	0.5
Iodized salt	0.2	0.2
Vit. A and D feeding oil	0.3	0.3

The 16 hens used in the experiment were selected because of their low hatchability records and were divided on the basis of these records into 2 comparable groups of 8 birds each. Their average hatchability from the start of egg production to 10 months of age was 54%. This ranged between 34 and 67% for individual hens. The object of the careful selection was to minimize the number of birds and hence the quantity of concentrate used. The hens were kept in a laying battery in an air-conditioned room for the duration of the experiment. Fertile eggs for hatchability studies were obtained by artificial insemination with semen from Barred Plymouth Rock males.

Both groups were fed diet 311 except that the diet of one of the groups was supplemented with 0.1% of the acid insoluble fraction of an extract of cow manure (Rubin and Bird[4]). This supplement was known to produce optimum growth in chicks when fed as 0.05% of an all-plant-protein diet containing 35% of soybean oil meal. The experiment was started March 6 and terminated November 12, 1946. After the groups had been on their respective diets for 6 weeks, the diets were reversed. This dietary regimen was

[1] Whitson, D., Titus, H. W., and Bird, H. R., *Poultry Sci.*, 1946, **25**, 52.

[2] Whitson, D., Titus, H. W., and Bird, H. R., *Poultry Sci.*, 1946, **25**, 143.

[3] Bird, H. R., Rubin, M., Whitson, D., and Haynes, S. K., *Poultry Sci.*, 1946, **25**, 285.

[4] Rubin, M., and Bird, H. R., *J. Biol. Chem.*, 1946, **163**, 393.

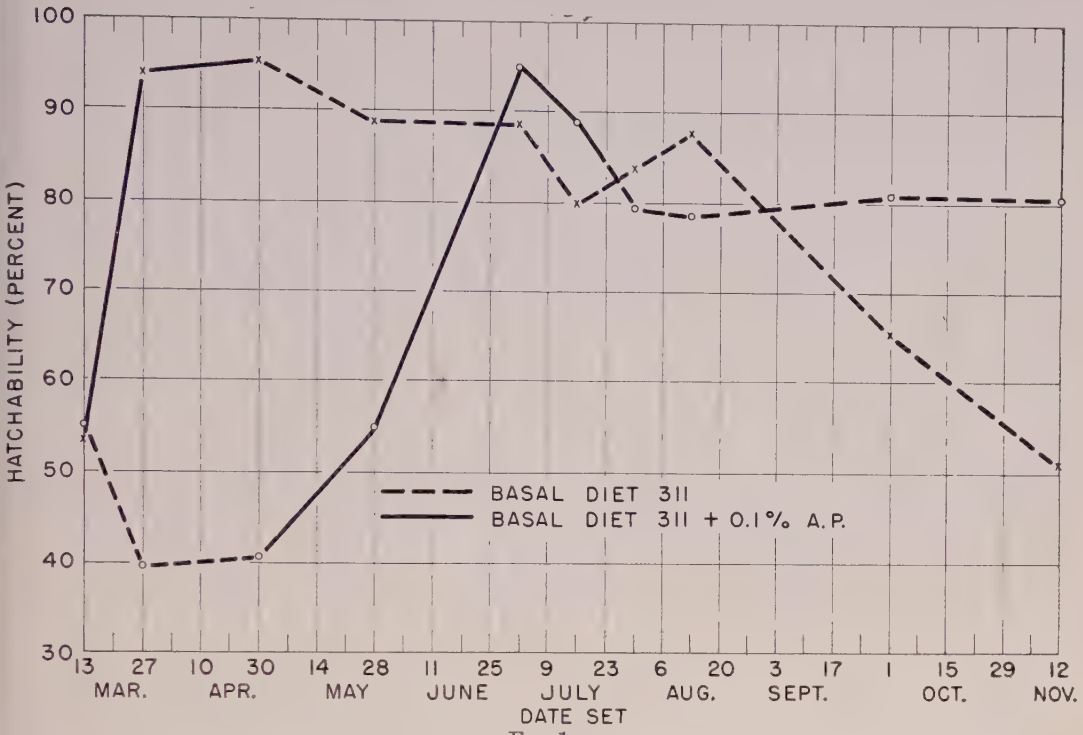

FIG. 1.

Effect upon hatchability of supplementing basal diet 311 with 0.1% of the acid precipitate of the water extract of cow manure.

continued until the seventeenth week of the experiment. At this time and until the termination of the experiment, all the hens were fed diet 311. The dietary changes and the hatchability data are illustrated in Fig. 1.

The concentrate prepared from cow manure brought about a rapid increase in the percentage of hatchable eggs, (Fig. 1), while the hens on the unsupplemented diet continued at a low level of hatchability. When these hens received the concentrate after the initial 6 weeks' period, their percentage of hatchable eggs also increased to a high level. The data in Fig. 1 show that when the supplement was removed from the diet, enough of the hatchability factor had been stored by the hens to enable them to maintain hatchability at a high level for 15 to 16 weeks. The experiment was terminated before the hens in the second group, which had received the concentrate for 11 weeks, had enough time to exhaust their store of the hatchability factor, even though they were fed the diet without the supplement for 16 weeks.

The hens used in part B were crossbreds (Rhode Island Red X Barred Plymouth Rock). During the growing period they had been fed a mash which contained 2 to 3% of fish meal and which supported rapid growth. They had access to grass range during the first 5 months of life. For the following 21 months, until June 4, 1946, they were fed diet 312 (Table I).

On June 4, 45 of these hens were changed to diet 311. They were housed in 2 colony laying houses with New Hampshire males and all eggs with good shells were incubated to determine hatchability. The experiment was terminated August 27, 1946.

During the 5 month period when the hens were fed diet 312 containing fish meal, the average hatchability was 82% of fertile eggs set. Hatchability during the 3 months period in which the hens were fed diet 311 was also 82% (Fig. 2).

Bird *et al.*[5] found that pullets had no

[5] Bird, H. R., Rubin, M., and Groschke, A. C., *J. Nutrition*, 1947, **33**, 319.

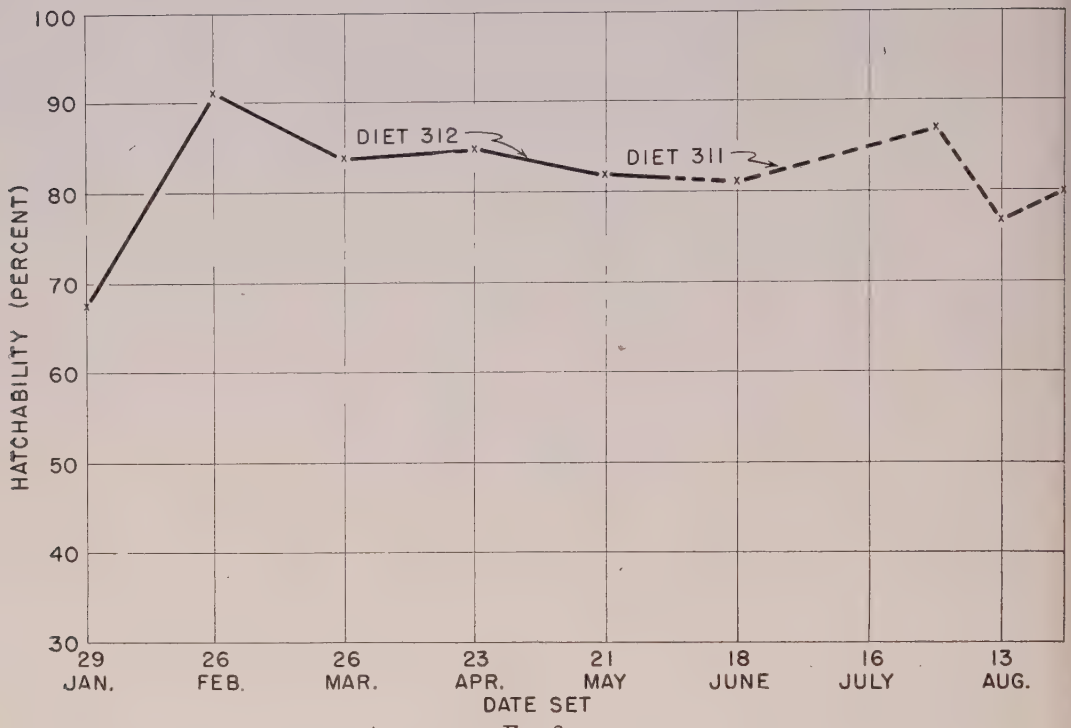

FIG. 2.

Effectiveness of bodily stores in maintaining hatchability in spite of dietary deficiency of the factor found in cow manure and in fish meal.

measurable stores of the hatchability stimulating factor at the time they began to lay. The pullets were of the same breeding as the hens in Part B of this experiment and like them had received a diet containing 2 to 3% of fish meal during the growing period. In this experiment hens fed 10% of fish meal or 0.1 percent of the concentrate from cow manure stored considerable quantities of the factor. Differences in the quantity of intake may account for the different results, or it may be that the mature bird has greater storage capabilities than the growing bird.

Summary. An acid precipitate of a water extract of cow manure which was highly potent as a source of the chick growth factor was shown to possess a high potency of the hatchability factor required by hens fed an all-plant-protein diet. This result is in accord with the view that the same dietary factor influences both growth and hatchability.

Hens that obtained the hatchability factor from the above mentioned concentrate or from fish meal, stored sufficient quantities of the factor, so that high hatchability was maintained for at least 12 to 15 weeks even when a deficient diet was fed.

15975

A Simplified Method for the Quantitative Determinations on Free Pregnanediol Excretion in Pregnancy.

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Venning and Browne[1] described a method for the isolation of sodium pregnanediol glucuronidate in the urine and theorized that this chemical substance represented the end-product in the metabolism of progesterone. Further studies confirmed the relationship of pregnanediol excretion to the luteal phase of the ovarian cycle and to normal pregnancy. Furthermore, a correlation between the administration of progesterone and the excretion of pregnanediol has been established although in the non-pregnant individual only a small fraction of progesterone administered can be recovered as sodium pregnanediol glucuronidate.

The gravimetric method of Venning for the quantitative estimation of pregnanediol is long and tedious and it has the disadvantage that a number of factors other than progesterone influence the excretion of glucuronidate. Astwood and Jones[2] described a method for the determination of free pregnanediol utilizing the hydrolysis of glucuronidate. Talbot *et al.*[3] made use of the color reaction produced by pure sulphuric acid to provide a colorimetric method for the quantitative determination of pregnanediol. Guterman[4] modified the Astwood-Jones method to provide for the more rapid qualitative determination of pregnanediol and made it more applicable to clinical use.

The quantitative method described here has the advantage of simplicity thereby making it possible to follow patients over long periods of time by serial determinations permitting the study of the complications of pregnancy and the evaluation of their therapy. The determination of free pregnanediol rather than the conjugated sodium pregnanediol glucuronidate avoids the danger of loss incurred during the urine collection period. Furthermore, other substances than pregnanediol are found in the urine as glucuronidates interfering with the accuracy of the determinations.

During the past year we have carried out over 1500 determinations of pregnanediol in the urine in about 100 patients. Some of these women had normal pregnancies and serial determinations were made throughout their pregnancies and for a week following their deliveries. These women served as normal controls to evaluate the method and to establish basic curves of pregnanediol excretion. The majority of the women had pregnancy complications in whom it was desired to follow the pregnanediol excretion in order to determine any variations from the normal curves in an attempt to establish the role of progesterone metabolism in these complications. In many of these patients daily determinations were possible but in the majority the collections of urine were made 2 and 3 times a week. All of the determinations have been made in duplicate to decrease the likelihood of error in the method.

Method. The women studied for the most part were out-patients who visited this clinic. They were given standard containers for urine collection and were carefully instructed to insure complete 24 hour samples. The first morning specimen on the day of collection was discarded and all urine throughout the day and

* This work has been done under a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

1 Venning, E. H., *J. Biol. Chem.*, 1937, **119**, 473.

2 Astwood, E. B., and Jones, G. E. S., *J. Biol. Chem.*, 1941, **137**, 397.

3 Talbot, N. D., Berman, R. A., MacLachlan, E. A., and Wolfe, J. K., *J. Clin. Endocrinol.*, 1941, **1**, 668.

4 Guterman, H. S., *J. Clin. Endocrinol.*, 1945, **5**, 407.

including the first morning specimen the next day were pooled.

The completed collection was brought to the laboratory the same morning and the determination started immediately. All examinations were made the same day they were received. No preservatives were used. In some cases patients were brought into the hospital and urine collections made largely by the patients themselves. It has been our experience that much more accurate 24 hour samples were made by cooperative patients than when collections were left to an already overworked nursing staff. Patients were carefully instructed to report any loss of part of the collections. Samples which for some reason were not complete were discarded. Determinations were made at weekly, biweekly and in some of the more interesting cases, daily intervals.

The chemical procedure employed is essentialy that of Astwood and Jones[2] as modified by Guterman.[4] We have in addition made a few changes of our own for the purpose of increasing the accuracy of the quantitative determination. The technique briefly is as follows:

1. One hundred cubic centimeters of urine, 50 cc of C.P.Toluene, 10 cc of conc. HCl and a few glass beads are added to a 500 cc flat bottomed Florence flask. The flask is connected by a one-holed cork stopper to a vertical reflux condenser and the contents boiled vigorously for 15 minutes on an electric hot plate.

2. The flask it then cooled under tap water to room temperature and its contents transferred to a 500 cc separatory funnel. The lower urine-acid layer is drawn off. The urine-acid layer is shaken twice with 10-15 cc volumes of fresh toluene and returned to the separatory funnel, the urine-acid layer being drawn off between each shaking.

3. The toluene emulsion layer in the separatory funnel is then washed twice with 15 cc portions of 0.1 N NaOH followed by 2 washings with 15 cc portions of distilled H_2O.

4. The washed toluene and toluene water emulsion layers are transferred to a 125 cc Erlenmayer flask. A few glass beads are added. The separatory funnel is rinsed with fresh toluene and the rinsings are added to the flask.

5. The mixture is boiled on an electric hot plate under a hood and when the emulsion layer has disappeared and the toluene is boiling smoothly 10 cc of freshly prepared 2% NaOH in absolute methanol are added slowly. The mixture is boiled until a granular precipitate is obtained and the solution has a yellow or greenish yellow appearance.

6. The toluene mixture is then filtered through fritted glass filters of medium porosity using slight suction. The precipitate is washed with the fresh hot toluene used to rinse out the flask.

7. The combined filtrates are evaporated in a hood utilizing a hot plate. The last traces of toluene are eliminated by means of an air stream. This avoids charring the residue.

8. Five cc of acetone are then added to the residue and the measure gently heated until solution is complete. Twenty cubic centimeters of 0.1 N NaOH are slowly added while the flask is still on the hot plate. When boiling occurs the flask is placed in the refrigerator overnight.

9. The precipitate that forms is collected by filtering through a fritted glass filter and washed with the rinsings of the flask using distilled H_2O. The precipitate is then washed with 10 cc of petroleum ether.

10. The receiving flask is changed and the precipitate dissolved by passing 10 cc of hot absolute ethanol through the funnel. If the precipitate shows any discoloration of a reddish or yellowish tint it is reprecipitated by adding 40 cc of distilled H_2O to the alcohol solution and heated to boiling. This last step is repeated until the precipitate is white.

11. The alcoholic filtrate is evaporated to dryness on a hot plate utilizing an air jet to remove the last few cubic centimeters.

12. Ten cc of C.P. H_2SO_4 are added to the dried white precipitate and allowed to stand one hour for full color development. After proper dilution (usually 1-10) the solution is read in a Coleman model 11 Spectrophotometer at a wave length of 420μ. The readings are interpolated on a curve made by using pure pregnanediol.

In order to test the accuracy of our quan-

TABLE I.
Comparison of Venning Gravimetric Method and the Modified Guterman Method.

No.	Pregnanediol	NaPG as Pregnanediol
1	78.1	78.3
2	68.4	60.1
3	65.7	67.9
4	18.2	16.3
5	9.1	8.1

titative results a few determinations using our technique and the original Venning method were run on the same samples. Fig. 1 shows the close agreement between the two methods.

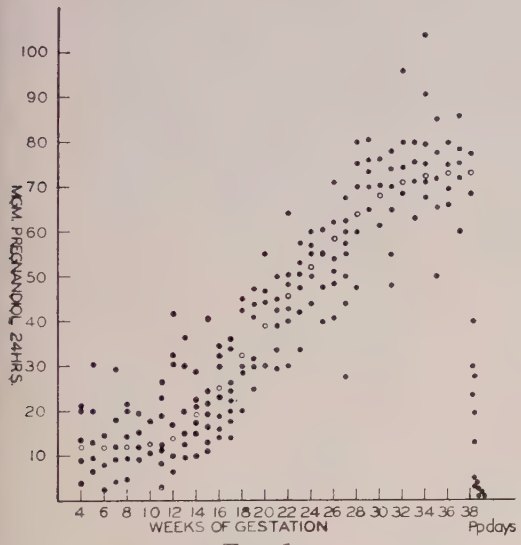

FIG. 1.
A composite curve demonstrating the excretion of pregnandiol in a group of patients during normal pregnancy and the immediate postpartum period.

Normal Pregnancy Curve. There is considerable variation in the daily output of pregnanediol in the individual with a normal pregnancy but the curve follows a typical pattern. During the first 12 to 14 weeks of the gestation the daily excretion will vary from a low of 5 or 6 mg to a high of 20 mg. Some individuals excrete more pregnanediol throughout the pregnancy than do others. The daily output increases slowly during the second trimester of pregnancy leveling off at about the twenty-eighth week of the gestation. The level remains fairly constant during the last 10 weeks of the pregnancy, varying from 70 to 90 mg per day. Individual daily amounts may fluctuate rather widely reaching as high as 120 mg or as low as 50 or 60 mg. Serial determinations on the same patient are extremely important for only in this way can one recognize sudden changes in the normal pattern. No sudden drop in the level of free pregnanediol prior to the onset of labor has been recognized in our curves although there may be some recognizable decrease in the daily output during the last few weeks prior to the onset of labor.

Following delivery there is a sudden drop in the excretion of pregnanediol. The first 24 hour urine collection may contain as little as half the amount present on the previous day. No more than 10 to 20 mg are eliminated during the second 24 hours. Small amounts varying from 2 to 3 mg may be present for an additional day or two, following which little is excreted. We have collected all urine in our postpartum patients by means of an inlying catheter to provide for complete samples. (Fig. 1)

Most authors agree that pregnanediol is the urinary metabolite of progesterone. During the latter half of the menstrual cycle and the early part of pregnancy, the corpus luteum is the principal source of this hormone. However, with the development of the placenta this organ becomes the principal source of the progestational hormone. It may serve as the only source for in 2 instances in our series as well as in the experience of others (Seegar and Delfs)[5] the removal of the ovary containing the corpus luteum did not alter appreciably the excretion of pregnanediol.

Placental functions must be directly related to the quantitative production of progesterone and the output of the biologically inert steroid pregnanediol. In that the placenta is primarily a circulatory organ providing the circulation link between mother and fetus, the excretion of pregnanediol depends indirectly on the efficiency of placental circulation. Complications of pregnancy associated with disturbances of placental function must invariably be reflected in disturbed pregnanediol excretion. It is not surprising that decreased amounts have been reported in patients who

[5] Seegar, G. E., and Delfs, E., *J. A. M. A.*, 1940, **115**, 1267.

threaten to abort, in the toxemias of pregnancy, in premature labor, in late death of the fetus. Before intelligent therapy of these complications can be developed, the relationship of progesterone metabolism to these conditions must be understood.

Summary.—A rapid, accurate colorimetric method for the quantitative determination of pregnanediol based on the methods of Venning, Talbot and Guterman is described. Serial determinations in normal pregnancy and pregnancy complications have been carried out in over 100 patients. Pregnanediol excretion in the last 28 weeks of the gestation can be used as a quantitative measure of uteroplacental circulation. During this period the placenta is the chief source of this urinary metabolite for corpus luteum activity wanes rapidly after the first trimester. Serial determinations in normal pregnancy and the complications of pregnancy may throw light on the adequacy of the placenta as the essential organ for the survival of the fetus.

15976

Symmetrical Patterns of Bacteriophage Production.

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In a previous note[1] it was shown that the problem of how bacteriophages are produced can be directly approached through the electron microscopy of metal shadowed "replicas" of the surfaces of agar cultures on which bacteria and bacteriophage are growing together. Such studies of several different bacteriophages are showing many of the phenomena involved in the production of these virus-like objects. The processes are complex and depend not only on the type of bacterium and the strain of bacteriophage but also on such factors as the rate and the duration of growth of the culture. Detailed evidence furnished by the electron microscope will be described elsewhere.

One of the most impressive aspects of the development of bacteriophage from bacterial protoplasm is its completeness, and in certain instances its regularities. With the T3 bacteriophage against the colon bacillus, the pattern of this conversion shows a symmetry as perfect as that of the molecular particles in a crystalline array[2] (Fig. 1). As in this figure the pattern often covers the entire surface of a bacterium; but it is also to be seen spreading throughout extruded protoplasm (Fig. 2). It extends into and includes the thick masses which were bipolar bodies in the original cells. When most clearly visible, the pattern is one of concavities but in many places the separate indentations are filled with spherical bodies having the size of free bacteriophage particles. Photographs have been obtained of this honeycomb structure starting to form within cells that otherwise seem normal; but it is often difficult to be sure whether these indentations are places where fully formed particles have escaped or where relatively immature particles are just beginning to form.

Correspondingly complete conversion to bacteriophage has been observed with other strains but highly symmetrical nets of particles have not been seen following infection with any of the "tailed" bacteriophages.

The many electron micrographs already made suggest far-reaching speculations into the nature of bacteriophages and of any viruses that may exhibit similar mechanisms of increase; but the techniques being used offer opportunities for gaining further information that make discussions of this nature seem premature.

1 Edwards, O. F., and Wyckoff, R. W. G., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 16.

2 Price, W. C., and Wyckoff, R. W. G., *Nature*, 1946, **157**, 764; Markham, R., Smith, K. M., and Wyckoff, R. W. G., *ibid.*, 1947, **159**, in press.

FIG. 1.
A chromium-shadowed electron micrograph of a colon bacillus infected with the T3 strain of bacteriophage. The regular pattern of evanavities extends over the entire surface of the organism. Magnification ca. 40,000.

FIG. 2.

A region of bacterial protoplasm left by the lysis of colon bacilli infected with the T3 strain of bacteriophage. An intact bacterium lies to the left of center of the field. Magnification *ca.* 30,000.

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Gaseous Distention in the Obstructed Small Intestine of Cats.

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In a previous paper[1] we demonstrated that sulfathalidine and sulfamethazine exert an inhibiting effect on gas production in the obstructed small intestine of the cat. This report concerns similar studies of other sulfonamides and of penicillin and streptomycin.

The following drugs were investigated: Sulfadiazine, sulfamerazine, sulfathiazole, sulfasuccidine, penicillin and streptomycin. Phthalic acid and succinic acid were tested because they are hydrolytic products of sulfathalidine and sulfasuccidine.[2] Additional experiments with sulfathalidine and sulfamethazine in smaller doses were carried out and are compared with the previously reported results with the larger doses.

Method. The experimental method is identical with that used previously. Cats weighing 1½-3 kg, which had been deprived of food and water for 24 hours, were subjected to laparotomy with the usual sterile precautions. A heavy cotton ligature was used to completely occlude the esophagus at the cardio-esophageal junction. A similar ligature was applied to the ileum just proximal to the ileocecal junction, thus producing a closed loop. Although the stomach was free of solids or liquids, gas up to 30 cc was present occasionally. This was aspirated prior to injection of the material to be studied. Two teaspoonfuls of powdered malted milk* in 100 cc of milk, with or without the drug to be tested, was injected into the stomach. The dose of sulfonamide was 1.5 g/kilo in most experiments; in the remaining ones, 0.5 g/kilo. The dose of penicillin was 30,000 U/kilo and of streptomycin 60,000 U/kilo. After 22-26 hours the animals were sacrificed by etherization and the amount of gas and liquid or semisolid contained in the closed loop was measured.

A total of 178 cats were studied. Six cats, not included in the results, were found dead after 24 hours. Most of these had received no drug. They showed enormous gaseous distention as the probable cause of death.

Results. Table I lists the average amounts of gas found and also the greatest and smallest amounts, to demonstrate the range of effectiveness of the various drugs used. No relation whatever was apparent between the amount of gas and the amount of solid and liquid material.

The data show that sulfadiazine, sulfamerazine, sulfamethazine, sulfathiazole and sulfathalidine are equally efficacious in depressing gas formation from malted milk in a closed loop. Doses of 0.5 g/kilo are just as effective as the larger dose. Both doses are large compared to amounts used clinically. Sulfasuccidine is definitely less effective, though significantly better than the controls. Succinic and phthalic acid have no effect whatever on gas production. Of the drugs studied, penicillin proved to be the most powerful inhibitor of gas formation. Streptomycin, on the whole, showed a good depressing effect, but the amounts of gas varied over a wide range.

Discussion. Since the sulfonamides in general exert their action on the gram negative bacteria of the intestinal tract (coli-aerogenes group) and on the clostridia normally present there, it is not surprising that these drugs work very much alike in depressing gas formation.

Of the 2 conjugated sulfonamides studied sulfathalidine exerts a definite antibacterial action *in vitro* and in the gut[2,3] while sulfa-

1 Segel, A., Schweinburg, F., and Fine, J., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 17.

2 Poth, E. J., and Ross, C. A., *Texas Reports on Biol. and Med.*, 1943, **1**, 345.

* Wander Powdered Malted Milk.

TABLE I.

Drug	Dose, g/k	No. of cats	cc gas		
			Max.	Min.	Avg
Sulfadiazine	1.5	10	35	4	14.3
"	.5	10	32	6	16.9
Sulfamerazine	1.5	10	30	1	12.8
Sulfamethazine	1.5	10	30	0	12.0
"	.5	4	14	8	10.0
Sulfathiazole	1.5	6	22	7	12.0
"	.5	12	25	1	11.1
Sulfathalidine	1.5	20	60	0	17.5
"	.5	4	31	5	15.2
Phthalic acid	.6	5	210	87	155.0
Sulfasuccidine	1.5	12	175	13	44.0
Succinic acid	.5	5	164	62	101
Penicillin	30.0 U/kg	10	38	0	8.5
Streptomycin	60.0 U/kg	29	225	0	33
Controls	—	31	215	55	117

succidine does not act *in vitro* but acts in the gut only after hydrolysis.[4] This fact explains the less effective action of sulfasuccidine. Phthalic and succinic acid exert no inhibitory action.

Since penicillin has no effect, except in excessive doses, on gram negative bacilli, it acts presumably by suppressing the clostridia which are present in the gut.

Since coliform bacteria predominate and clostridia are relatively few, one would expect gas formation to be suppressed to a lesser degree by penicillin than by those drugs which work on both clostridia and gram negative bacilli. This is not the case, however, since it works best of all the drugs studied.

The results with streptomycin are difficult to explain. The range of gas production is fairly narrow for all drugs except streptomycin. Of 29 cats treated with streptomycin, 16 had either no gas or less than 5 cc; 6 had less than 25 cc; 3 showed amounts of gas between 25 and 100 cc and 4 had 110, 150, 195, and 225 cc of gas respectively. The last of these is a little more than the highest amount of gas found in the controls, or with phthalic and succinic acid, which may also be considered controls. We are not able to explain these observations.

A study of the chemical composition of the gas and the associated changes in the bacterial flora of the small intestine of dogs brought about by these drugs is proceeding.

Conclusions. Gas formation from malted milk in milk in a closed loop, comprising the stomach and the entire small intestine, is markedly depressed by sulfadiazine, sulfamerazine, sulfamethazine, sulfathiazole and sulfathalidine. There is a definitely less, but still significant depression by sulfasuccidine. Phthalic and succinic acid have no inhibitory effect on gas production. Streptomycin works well in the majority of the experiments, but is a complete failure in others. Penicillin works best of all the drugs tested.

We wish to thank Miss Sunya Gordon and Mr. Thomas Barnett for valuable technical assistance.

[3] Schweinburg, F. B., and Yetwin, I. J., *J. Bact.*, 1945, **49**, 193.

[4] Poth, E. J., and Knotts, F. L., *Arch. Surg.*, 1942, **44**, 208.

15978

Urinary Excretion of Radioactive Iodine, I^{131}, in a Case of Severe Hyperthyroidism.*

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Earlier studies of Hamilton,[1] Hertz and Roberts,[2] Chapman and Evans[3] and others have provided helpful data with respect to the absorption of I^{130}, its deposition in the thyroid, elimination, and effectiveness in controlling the symptoms in patients suffering with hyperthyroidism. This isotope, obtained from the cyclotron, has a half-life of 12 hours. The isotope, I^{131}, which can be obtained carrier-free from the uranium pile has a half-life of 8 days. The irradiation is, therefore, distributed over a much longer time when I^{131} is used for therapeutic purposes. The amount of I^{131} required for treatment needs to be determined and little data is as yet available in the literature with respect to its use in the treatment of hyperthyroidism. The purpose of the present report is to provide data concerning the excretion and retention of I^{131}.

Radioactive iodine,† I^{131}, was administered orally to a female patient, age 29, suffering from a severe hyperthyroidism of diffuse type with marked exophthalmos. Lugol's solution had been given for a period of several months but had produced only brief improvement and was discontinued 3 weeks before the first dose of radioactive iodine was administered. The patient proved to be sensitive to thiouracil and she was not in good enough condition to tolerate surgery. Her weight had gone down to 87 pounds and the BMR was +70. The weight of the thyroid was estimated to be 50 g.

A dose of 9.1 millicuries of I^{131} was given on October 11, 1946. No toxic reaction was observed. Urinary excretion was determined by means of a beta-ray Geiger-Muller Counter.[4] In the first four 12-hour intervals it was respectively 10.4%, 15.5%, 10.4%, and 8.5% (in per cent of I^{131} given), thus indicating a total retention after 2 days of 54%. A dose of 3 millicuries was given on November 24, 1946. Urinary excretion in the first two 12-hour intervals was respectively (in per cent of I^{131} given) 12.7% and 1.8% giving a total retention after 1 day of 85.5%.

Calculated values of retention of iodine in the body are given in Table I, and measurements on excretion of I^{131} in the urine are given in Fig. 1. Iodine retention is given in per cent of the original dose. (Both radioactive decay and excretion factors being considered.) Excretion is plotted in per cent of the iodine retained at a time half-way between the collection of specimens.

The question has been raised of the advisability of giving stable iodine following the administration of the radioactive isotope. For this reason, stable iodine was administered as Lugol's solution for a period of 2 days after the urinary excretion of I^{131} had reached a low level. (The continued low level of excretion of I^{131} has been explained on the basis of decay of thyroxin in the blood.) Administration of Lugol's solution was followed, each time, by excretion of I^{131} in relatively large amounts. The excretion of radioactive iodine seemed to begin some 24 hours after the stable iodine was given. This delay is quite different from the immediate excretion (in the first 12-hour

* Aided by the Research Funds of the Graduate School of the University of Minnesota.

1 Hamilton, J. G., *Radiology*, 1942, **39**, 641.

2 Hertz, S., and Roberts, A., *J. Am. Med. Assn.*, 1946, **131**, 81.

3 Chapman, E. M., and Evans, R. D., *J. Am. Med. Assn.*, 1946, **131**, 86.

† The radioactive iodine was obtained through the cooperation of the Isotope Branch, Research Division, United States Engineering Office, Manhattan District, Oak Ridge, Tenn.

4 Wang, J. C., Marvin, J. F., and Stenstrom, K. W., *Rev. Scient. Inst.*, 1942, **13**, 81.

TABLE I.

Interval, days	% of administered I^{131} retained (at midpoint of interval) I—9.15 mc (100%) (.18 mc/g thyroid)	II—3.0 mc (100%) (.06 mc/g thyroid)	Equivalent roentgens per interval I	II
0 -0.5	93.5	91.5	1040	330
0.5-1.0	78	82	870	300
1.0-1.5	61	76	680	275
1.5-2.5	51	70	1140	510
2.5-3.5	43	64	960	470
3.5-4.5	38	58	850	425
4.5-5.5	34	51	760	370
5.5-6.5	30	43	670	315
6.5-7.5	24	37	535	270
7.5-8.5	17.5	33	390	240
8.5-9.5	13	30	290	220
Estimated additional dosage (extended over a period of several weeks)			1765	1775
Total dose in equivalent roentgens			9950	5500

interval) following administration of radioactive iodine. The same experiment was carried out in one case of carcinoma of the thyroid with metastasis (unpublished).)In this case, however, administration of Lugol's solution did not result in increased excretion of I^{131}.

The estimated dose in roentgens for each 12-hr or 24-hr period is given in Table I. This data was calculated in accordance with

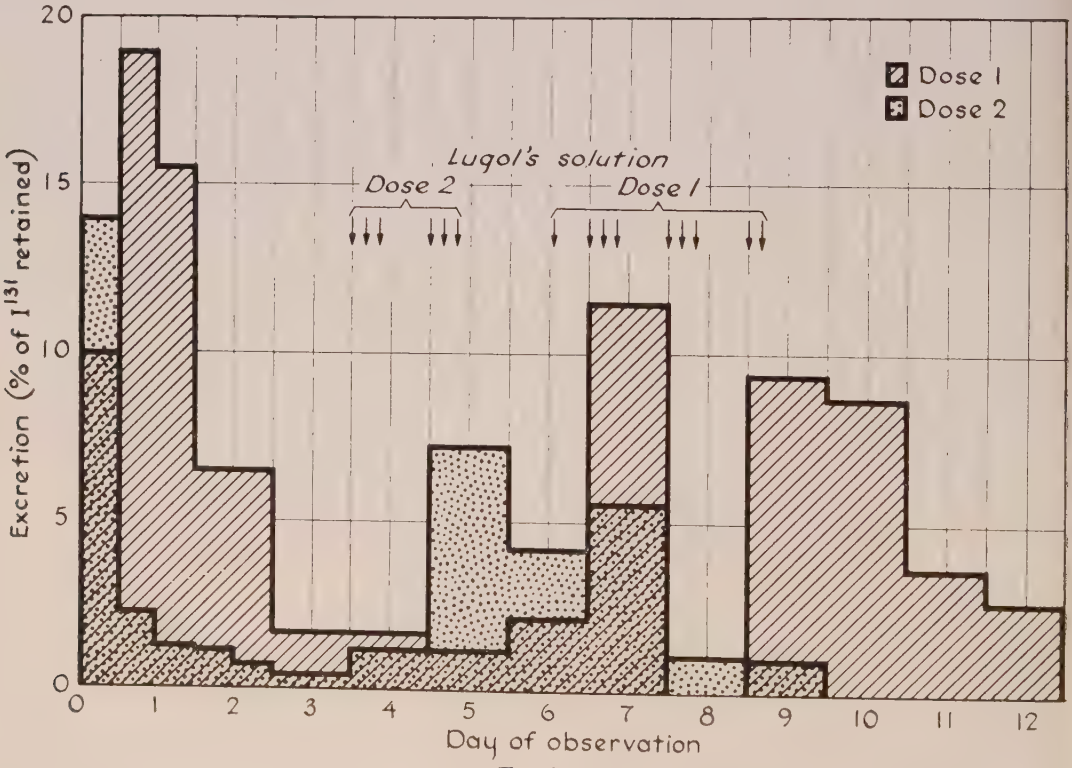

FIG. 1.

Urinary excretion of I^{131} per 12 hours calculated in per cent of iodine retained at a time half-way between the collection of specimens. Dose 1 was 9 mc. Dose 2 was 3 mc, one month later. No data were available for the 8th day following dose 1 as the specimen was lost. Arrows indicate the time of administration of Lugol's solution.

Evans[3] formula, on the basis of a thyroid weight of 50 g. (The table gives estimates of the dose per 12 or 24-hour interval and of the total dose). The total dose is estimated to be 9950 roentgens for the first administration of 9.1 millicuries, and 5500 roentgens for the second administration of 3 millicuries. It should be noted that the total dose in roentgens following the second administration (3 millicuries) agrees with the simplified formula of Hertz and Roberts,[2] roentgens eqivalent

$$= 117{,}000 \times \frac{\text{millicuries } I^{131}}{\text{grams thyroid}}$$

It is too early to evaluate the result of the treatment. Though the patient has improved somewhat and has gained weight, no decided reduction of the BMR occurred in the first 2 months following the first dose, and it seems probable that further treatment will be required.

Summary. Information is given of the urinary excretion of radioactive iodine following administration of relatively large doses in a patient with severe hyperthyroidism. Administration of stable iodine (in Lugol's solution) after the urinary excretion of I^{131} had reached a low level resulted in a pronounced increase in this excretion, which lasted some 48 hours after discontinuing Lugol's solution. This increase in excretion of I^{131} was not observed following administration of Lugol's solution in one case of carcinoma of the thyroid with metastasis. Retention of 85% of the I^{131} by the thyroid agrees with other figures on the retention of I^{130} by the hyperplastic thyroid, when iodine has been withheld for a period of over 4 weeks prior to radioactive iodine.‡

‡ A third dose of 9 millicuries of I^{131} was given the same patient in January, 1947. Loss of iodine in the first 2 days was high (50%). Use of Lugol's solution again resulted in increased excretion of radioactive iodine. The metabolic rate had returned to normal when the patient was next seen in March, 1947.

Two additional cases have since been treated with doses of 9 millicuries of I^{131} (estimated 50 grams thyroid tissue). Excretion was much the same as that following the second dose of iodine for patient No. 1. Use of Lugol's solution resulted in increased excretion of radioactive iodine in both of these cases.

Dr. C. J. Watson, Director of the Department of Internal Medicine, University of Minnesota Hospitals, made the arrangements for the treatment of the patient, and we are obliged to him for the opportunity to carry out these measurements and for valuable advice.

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The Development of *Fundulus heteroclitus* Embryos in Solutions of Metrazol.

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Speidel[1] has attempted to ascertain the specific effect of metrazol on the nervous system by immersing frog tadpoles in aqueous solutions of it and by studying, by direct microscopic and ciné-photomicrographic methods, the outgrowth of living nerve fibers in the tadpoles' tails. Using a 2% solution for approximately 20 minutes, Speidel found that the growing nerve endings degenerated during the period of immersion, but recovered and subsequently regenerated when the tadpoles were returned to pond-water.

The present communication reports the results of studying the development of cer-

* Aided by a grant from the Penrose Fund of the American Philosophical Society.

1 Speidel, C. C., *Proc. Am. Philos. Soc.*, 1940, **83**, 349.

tain aspects of gross behavior in whole embryos (*Fundulus heteroclitus*) which were immersed in solutions of metrazol at different stages of development and which remained in them for varying periods of time. The investigation was undertaken with the possibility in mind that subjecting embryos to treatment at various stages of development might produce alterations of structure and function in the central nervous system which could be correlated with the particular fiber-tracts being laid down during the period of treatment.

Over 500 eggs of *Fundulus heteroclitus* were treated with aqueous solutions of metrazol (Pentamethylentetrazol, Bilhuber-Knoll Corporation). Solutions, made in distilled water, were used in strengths of either 0.1% or 2%. The embryos were introduced into the solutions at stages varying between 6 and 34 (Oppenheimer[2]), and remained in them for periods varying from a few hours to 14 days. In some cases they remained in the solutions until fixation; in others they were transferred to tap- or sea-water. Some embryos were dechorionated before treatment, others were left in their chorions throughout; still others were dechorionated during the course of treatment or recovery. All embryos were observed at least twice daily; those which survived treatment were preserved and are being prepared for histological study.

The gross effects, which were more drastic in dechorionated embryos than in those with chorions intact, varied also in degree according to the age of the embryos, the strength of the solutions used and the duration of the experiments. In some cases recovery occurred after the embryos were transferred to tap- or sea-water.

None of the embryos treated with 2% metrazol solution before gastrulation developed normally; some of those treated at pregastrular stages with 0.1% solution reached stage 32, the stage of hatching, and completed the major part of their early development. Embryos treated subsequent to gastrulation but before the establishment of circulation developed for as long as 14 days in the 0.1% solution; of comparable embryos treated with 2% solution, only 2 (both left in their chorions during treatment and transferred from metrazol after 2½ days), reached stage 31

There was a marked effect on the circulation of embryos treated between stages 23 and 32; the rate of the heartbeat became markedly slowed, and a large vesicle, filled with static blood, formed just distal to the sinus venosus. In addition, the tonic relationships of the longitudinal body musculature were often affected in embryos treated at these stages. Many of the embryos treated between stages 23 and 26 exhibited sharp localized contractures of the trunk musculature. In addition, many embryos treated between stages 23 and 32, whether or not such localized contractures were present, exhibited various degrees of kyphosis or lordosis which involved sometimes trunk, sometimes tail, sometimes both. Embryos treated between stages 27 and 32 in some cases also showed abnormalities in the action of the mouth and opercular apparatus, which were held open without performing the usual rhythmic movements.

Embryos treated subsequent to stage 32 did not exhibit the same tonic abnormalities which characterized the group just described and were not kyphotic or lordotic. They sometimes, however, in contrast, exhibited sharp lateral contractions and held their tails bent to left or right. Their mouths were almost invariably stretched wide open and exhibited no rhythmic movements. Righting reflexes were lost in these embryos during the period of treatment.

Whether the effect of the drug on the reactions of the embryos acts through the nervous system or directly on the muscular system, and whether or not the drug has resulted in morphological changes within the nervous system that do not produce functional changes in behavior, can be ascertained only after histological examination of the embryos is completed. It is clear, however, that subjecting whole embryos to the action of the drug provides a further method for study of the development of structural and functional relations in the embryonic central nervous system.

[2] Oppenheimer, J. M., *Anat. Rec.*, 1937, **68**, 1.

15980

Effects of Testis Hyaluronidase and Seminal Fluids on the Fertilizing Capacity of Rabbit Spermatozoa.

M. C. Chang. (Introduced by D. Rapport.)

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An enzyme, extracted from mammalian testis, which increases the permeability of skin and other tissues containing hyaluronic acid is known as hyaluronidase.[1-4] The cumulus cells surrounding the tubal ova of the rabbit were shown to be dispersed in the presence of fairly large numbers of sperms by a heat labile enzyme,[5,6] now identified as hyaluronidase.[7-10] On the basis of these observations, the participation of hyaluronidase in fertilization,[7-10] the capacity of hyaluronidase to increase the fertilizing power of sperms,[11] and the clinical use of hyaluronidase for sterility[12] have been reported. Rowlands' report[11] on increased fertilizing capacity in rabbits was based on the use of heated seminal fluid which contains hyaluronidase. In the following experiments we have examined the effects of purified testis hyaluronidase on fertilization *in vivo*.

Methods. Adult non-pregnant doe rabbits were superovulated according to Pincus.[13] They were inseminated with a minimal effective number of spermatozoa (number of sperms needed to fertilize only a small number of ova) suspended in different fluids just before the intravenous injection for the induction of ovulation. The rabbits were sacrificed 25 to 30 hours later and the ova were flushed out from the oviducts. The fertilized and unfertilized ova were counted.

The semen of a single male rabbit was collected with an artificial vagina[14] for insemination in order to control the variation of sperm quality between different individuals. The interval between each collection of sperm was 3 to 4 days in order to keep the sperm quality constant. The general method of insemination was carried out according to Walton.[15,16] The semen just after collection was diluted with saline (0.9% NaCl) about 1 part to 1,000. Then the sperm concentration was immediately counted by means of the hemocytometer technique. Saline or sperm was added if the concentration of sperms was too high or too low. Then 0.5 ml of this suspension was added to one of the following: (1) 0.5 ml of saline containing a known amount of hyaluronidase, (2) 0.5 ml of supernatant fluid of normal semen after heating, (3) 0.5 ml of semen from a vasectomized male, (4) 0.5 ml of saline serving as a parallel control. These mixtures (all 1 ml in volume) were introduced into the vagina of each rabbit. Usually, 6 rabbits were inseminated at a time. The time interval between collection of semen and the first insemination was about 10 minutes, and that between the first and last insemination was about 20-30 minutes.

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4 Chain, E., and Duthie, E. S., *Brit. J. Exp. Path.*, 1940, **21**, 324.
5 Pincus, G., and Enzmann, E. V., *J. Exp. Med.*, 1935, **62**, 665.
6 Pincus, G., and Enzmann, E. V., *J. Exp. Zool.*, 1936, **73**, 195.
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12 Kurzrok, R., Leonard, S. L., and Conrad, H., *Am. J. Med.*, 1946, **1**, 491.
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15 Walton, A., *Proc. Roy. Soc. B.*, 1927, **101**, 303.
16 Walton, A., *J. Exp. Biol.*, 1930, **7**, 201.

The testis hyaluronidase used was obtained from bull testicles (Schering Corporation). The seminal hyaluronidase was prepared according to Rowlands;[11] *i.e.*, normal semen was heated at 50° C for 8 to 11 minutes to kill sperms, and then kept on ice for 5 minutes and centrifuged. The supernatant fluid was used. After insemination, the heated seminal supernatant fluid and the vasectomized male semen were assayed by the viscosimetric method[17] and expressed as mg of testis hyaluronidase. The number of spermatozoa in the suspension was counted again 4 to 6 times and the average number of sperms inseminated was calculated.

Results. The complete data of these experiments are presented in Table I. It is evident that the fertilizing capacity of spermatozoa was not affected when testis hyaluronidase (1 to 0.65 mg per ml) was added. The average percentage of fertilized ova for 10 experimental does was 30 and that of 10 parallel control does was 27. The total number of fertilized ova in the experimental group was 70 out of 203 (34%), while that in the control group was 48 out of 198 (24%).

The average percentage of fertilized ova (60%) in the 13 experimental does inseminated with supernatant fluid of normal semen which contained hyaluronidase (1.05 to 0.2 mg per ml) was higher than that of 13 parallel control does (38%). But there is no statistical significance of the difference ($t=1.56$, $P<0.2>0.1$). However, the total number of ova fertilized in the experimental group (62%) is higher than that of the parallel control group (39%).

The average percentage of fertilized ova in those 10 does inseminated with sperms suspended in saline and the semen of vasectomized bucks which contains no hyaluronidase was 63, while that of 9 parallel control does was only 15. The difference is statistically significant ($t=4.66$, $P<0.01$). The total number of ova fertilized in the experimental group (65%) is higher as compared with that in the parallel control group (21%).

It is quite clear from these data that the extra hyaluronidase added to the sperm suspension does not influence the fertilizing capacity of spermatozoa. On the other hand, seminal fluid with or without hyaluronidase does increase the fertilizing capacity of rabbit spermatozoa.

Discussion. It is a common thought that seminal fluid is not important for the fertilizing capacity of spermatozoa because the epididymal spermatozoa,[15,16,18] spermatozoa separated from seminal fluid by centrifugation,[19] and semen in a very diluted form[20,21] are able to insure fertilization. The present investigation, however, reveals clearly that the importance of seminal fluid shows up when the number of spermatozoa is decreased to a minimum. Thus, any disturbances of accessory glands may affect the fertility of a male though clinical data on this point are still scarce.[22]

The great variation in the percentage of fertilized ova per doe (Table I) under strictly controlled experimental conditions leads one to reject those positive conclusions based upon only a few clinical cases or based on some experimental studies without strict control of variations of sperm quality in different individuals and in different time intervals of collection in the study of such an intrinsic process as fertilization.

Although the dispersal of cumulus cells surrounding the ovum by sperms or by hyaluronidase *in vitro* is unquestionable, the role of hyaluronidase in the complicated process of fertilization *in vivo* is still uncertain. Even if hyaluronidase *per se* plays an important role in fertilization, the hyaluronidase of spermatozoa is quite adequate to perform its function without further addition of hyaluronidase.

Summary. Thirty-three doe rabbits were inseminated with a minimal effective number of spermatozoa suspended in saline containing

[17] Hadidian, Z., and Pirie, N. W., *Biochem. J.*, in press.

[18] Young, W. C., *J. Exp. Biol.*, 1931, **8**, 151.

[19] Walton, A., *Proc. Am. Soc. Ani. Prod., 31st Meeting*, 1938, 238.

[20] Chang, M. C., *J. Exp. Biol.*, 1946, **22**, 95.

[21] Salisbury, G. W., Elliott, I., and Van Demark, N. L., *J. Dairy Sci.*, 1945, **28**, 233.

[22] Huggins, C., *The Role of the Accessory Glands in Fertility. Diagnosis in Sterility.* 1946. Edited by E. T. Engle. Charles Thomas, Publisher, Ill.

TABLE I. Effect of Hyaluronidase and of Seminal Fluid on the Fertilizing Capacity of Rabbit Spermatozoa.

Experimental series	Exp. No.	No. of sperms inseminated × 1000	Experimental group (E) Does No.	Conc. of hyaluronidase mg/ml	E: No. ova Fertilized	E: Unfertilized	E: Total	E: % fertilization	Control group (C) Does No.	C: No. ova Fertilized	C: Unfertilized	C: Total	C: % fertilization	% difference in fertilization (E-C)
Testis Hyaluroni-	2	228	410	1	2	13	15	13	411	18	3	21	86	—17
			413		20	19	39	51	414	2	11	13	15	
idase added	3	226	416	,,	0	2	2	0	417	0	23	23	0	—15
			419		0	28	28	0	420	7	18	25	28	
	4	342	363	,,	13	0	13	100	365	0	27	27	0	27
			371		6	31	37	16	378	4	6	10	40	
	6	50	421	.65	23	6	29	79	423	2	11	13	15	48
			424		0	13	13	0	426	1	29	30	3	
	8	116	337	,,	0	11	11	0	434	7	13	20	35	—17
			436		6	10	16	38	437	7	9	16	44	
Avg % of fertilization per doe								30					27	3
Total No. of ova					70	133	203	34		48	150	198	24	10
Supernatant fluid	5	274	394	.625	20	0	20	100	393	23	0	23	100	— 2
			397		31	3	34	91	400	12	1	13	92	
of heated nor-	6	50	422	.20	0	18	18	0	423	2	11	13	15	1
			425		3	16	19	16	426	1	29	30	3	
mal semen added	7	111	472	.325	19	11	30	63	428	14	2	16	88	— 3
			431		5	5	10	50	432	0	6	6	0	
	8	116	433	.75	12	0	12	100	434	7	13	20	35	50
			435		5	2	7	71	437	7	9	16	44	
	9	96	444	.56	10	0	10	100	445	2	12	14	14	34
			446		1	12	13	8	447	1	7	8	13	
	10	130	456	.5	16	7	23	70	458	3	24	27	11	59
	11	173	460	1.05	10	6	16	63	461	1	18	19	5	— 3
			463		1	1	2	50	464	18	8	26	69	
Avg % of fertilization per doe								60					38	22
Total No. of ova					133	81	214	62		91	140	231	39	23
Semen from	10	130	454	.05†	16	0	16	100	455	0	11	11	0	78
			457		22	6	28	79	458	3	24	27	11	
vasectomized	11	173	459	0	11	8	19	58	461	1	18	19	5	16
			462		7	5	12	58	464	18	8	26	69	
bucks added	12	150	467	0	4	4	8	50	466	1	12	13	8	49
			469		24	8	32	75	468	1	26	27	4	
									470	13	19	32	41	
			508	0	19	8	27	70	510	0	16	16	0	53
	17	125	511		6	17	23	26	513	0	9	9	0	
			509*	0	10	3	13	77						
			512*		6	9	15	40						
Avg % of fertilization per doe								63					15	48
Total No. of ova					125	68	193	65		37	143	180	21	44

*Semen of vasectomized male heated at 50°C for 20 minutes was used. † Very few dead sperms present.

purified testis hyaluronidase, or saline and supernatant fluid of heated normal semen containing hyaluronidase, or in saline and semen of vasectomized buck containing no hyaluronidase. Thirty-two does were inseminated at the same time with the same number of sperms collected from the same rabbit but suspended in saline, serving as parallel controls. It was found that it was the seminal fluid, not hyaluronidase, which really increased the fertilizing capacity of spermatozoa.

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Range of Antibiotic Activity of Protoanemonin.*†

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Extracts of buttercups and *Anemone pulsatilla* exert an inhibitory action on the growth of a number of pathogenic bacteria.[1,2] The active principle extracted from *A. pulsatilla* proved to be protoanemonin.[3] The present studies deal with the titration of the antibiotic activity of protoanemonin when tested against a variety of bacteria and fungi, as well as a few representatives among the viruses and protozoa.

Methods of testing the bacteria. The protoanemonin used in these experiments was extracted from dried ground *A. pulsatilla* or was prepared synthetically, as described previously.[3] The stock was a 1-100 dilution by volume in sterile distilled water from which further dilutions in the test media were prepared.

The *in vitro* susceptibility of the bacteria, with the exception of the Mycobacteria, was determined by inoculating 0.5 cc of a 10^{-3} dilution of a 24- or 48-hour culture of the test organisms into 4.5 cc of media containing decreasing concentrations of the antibiotic. The media chosen, as indicated in the tables, were those which provided favorable growth conditions for the bacteria under investigation. In some cases the organisms were tested when grown in each of 2 media. After a period of incubation at 37°C, sufficient for optimum growth of the control tubes, containing no protoanemonin, the cultures were examined for the presence of visible turbidity or other evidence of growth, such as the production of a pellicle, pigment or gas. The maximum dilution of protoanemonin capable of preventing the appearance of growth in the period of time specified is recorded in the tables.

Testing of the Mycobacteria required modifications in technique. A loopful of pellicle 1 cm in diameter from a 25-day-old culture was used to inoculate 100 cc volumes of

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† We are greatly indebted to Drs. M. M. Steinbach and C. J. Duca for the tests with the Mycobacteria, to Dr. Rhoda Benham for the cultures of the fungi, to Drs. J. A. Dawson and G. W. Kidder for the cultures of the tetrahymena, and to Prof. Paul Brutsaert of the Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium, for first making the observations on the sensitivity of the protozoa to protoanemonin.

1 Seegal, B. C., and Holden, M., *Science*, 1945, **101**, 413.

2 Carlson, H. J., Bissell, H. D., and Mueller, M. G., *J. Bact.*, 1946, **52**, 155.

3 Baer, H., Holden, M., and Seegal, B. C., *J. Biol. Chem.*, 1946, **162**, 65.

TABLE I.

The Maximum Dilution of Protoanemonin Preventing the Visible Growth of a Number of Gram-positive Aerobic and Anaerobic Bacteria.*

Organism	Dilution of protoanemonin × 1000	Media used	Time of incubation
Strep. hem. Group A (3 strains)	52-66	1% chicken serum meat infusion broth	Overnight (approx. 16 hr)
Strep. hem. Group D (2 strains)	16-20	" " " " " "	"
Strep. viridans (2 strains)	33-55	" " " " " "	"
D. pneumoniæ Types I, II, III, VII	55	" " " " " "	"
Staph. Oxford H	60-83	Meat infusion broth	"
Staph. Oxford H	100-150	Casein hydrolysate broth	"
Staph. albus	66	Meat infusion broth	"
M. lysodeikticus	44	" " "	"
C. diphtheriæ	75-100	0.5% glucose meat infusion broth	"
C. hoffmanni	75-100	" " " " "	"
C. xerosis	6-12	" " " " "	"
C. xerosis	16-30	Casein hydrolysate broth	"
My. tuberculosis hominis	166	Sauton's	1 month
My. tuberculosis hominis	100-330	Dubos'	4 days
My. tuberculosis bovis	100-250	"	"
My. tuberculosis avium	250-450	"	"
B. subtilis (contains spores)	20-50	Meat infusion broth	Overnight (approx. 16 hr)
B. anthracis (contains spores)	20-50	" " "	"
Cl. histolyticus	30-350	0.1% agar in 1% glucose meat infusion broth	48 hours
Cl. tetani	100-120	" " "	"
Cl. novyi	30-60	" " "	"
Cl. welchii	30-350	" " "	"
Cl. oedematiens	50-100	" " "	"
Cl. sporogenes	30-350	" " "	"

* All organisms were tested repeatedly. When results varied from day to day the extremes of variation are given.

Sauton's media containing the appropriate dilutions of protoanemonin. When Dubos'[4] medium was used 0.1 cc of a 5-day-old culture was the inoculum employed to seed the 5 cc of test medium.

All tests were repeated at least once and usually several times. If the concentration of protoanemonin causing inhibition of growth varied on different days the range of activity is indicated in the tables.

Results with bacteria. In Tables I and II it may be seen that all the bacteria tested possessed some degree of sensitivity to the antibiotic action of protoanemonin. However, the maximum inhibiting dilution varied widely. One strain of *Corynebacterium xerosis* required a 1-6000 dilution to prevent growth, while the Mycobacteria were inhibited by approximately one-thirtieth of this amount of protoanemonin. It is interesting also that many of the Gram-positive bacteria were somewhat less sensitive that the Gram-negative organisms. Those organisms tested in both broth and casein hydrolysate medium[3] showed greated sensitivity to protoanemonin when grown in the semi-synthetic medium.

Experiments were undertaken to determine whether the acidity of the medium, the size of the inoculum and the age of the culture might be contributing factors in the susceptibility of bacteria to protoanemonin. First, the possibility that the acidity of the medium might influence the inhibiting action of protoanemonin was investigated, using *Escherichia coli* as the test organism and hydrogen ion values ranging from pH 6.7 to pH 7.8. In neither meat infusion broth nor casein hydrolysate broth, with or without the addition of 1% glucose, was there a change in the end titer of the protoanemonin.

The size of the inoculum was investigated, using 4 organisms, *Staphylococcus oxford* H, *Corynebacterium xerosis, Escherichia coli* and

[4] Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

TABLE II.

The Maximum Dilution of Protoanemonin Preventing the Visible Growth of a Number of Gram-negative Aerobic Bacteria. All Tests Were Incubated Overnight (Approximately 16 Hours).*

Organism	Dilution of protoanemonin × 1000	Media used
K. pneumoniæ	40	1% chicken serum meat infusion broth
N. catarrhalis	166	Glucose broth
Ps. aerugenosa (3 strains)	30-100	Meat infusion broth
" " " "	40-200	Casein hydrolysate broth
Ser. marcescens	80-180	Meat infusion broth
V. choleræ	60-100	" " "
" "	200	Casein hydrolysate broth
P. vulgaris	80	Meat infusion broth
P. OX19	120	" " "
Alk. fecalis (2 strains)	60-80	" " "
Es. communis	50	" " "
Es. communior	50	" " "
" "	100	Casein hydrolysate broth
Eb. typhi "O" and "H"	250-330	Meat infusion broth
S. paratyphii	166	" " "
S. Schottmülleri	166	" " "
Sh. dysenteriæ Shiga	250	" " "
" " Flexner	166	" " "
" " Sonne	166	" " "

* See footnote Table I.

Vibrio cholerae. Sixteen-hour cultures, undiluted and diluted 10^{-1}, 10^{-3} and 10^{-7}, were added to casein hydrolysate broth and to meat infusion broth, both media containing varying amounts of the antibiotic. The findings indicated that the antibacterial activity was independent of the size of the inoculum when the inoculum was diluted 10^{-1}, 10^{-3} or 10^{-7}, however the undiluted inoculum was not consistently inhibited by similar dilutions of protoanemonin.

The effect of the age of the inocula upon the susceptibility of the same 4 organisms was determined by using 2-, 4-, 6-, 16-, and 48-hour cultures. Each culture was diluted until the turbidity approximated that of the 2-hour culture, and 0.5 cc served as the inoculum. The number of viable organisms was determined by pouring plates from each tube and counting colonies. The variation in number of viable organisms was well within the limits of what was found to be without effect on the outcome of the test. The results showed that the end titer of protoanemonin was the same irrespective of the age of the inoculum used.

Methods and results with fungi. Three yeasts, a non-pathogenic *Saccharomyces cerevisiae* and the pathogenic *Candida albicans* and *Cryptococcus neoformans,* were grown in 1% glucose broth for 2 to 5 days. Five-tenths cubic centimeters of a 10^{-3} dilution of the culture was added to 4.5 cc of the glucose broth containing varying amounts of the antibiotic. The dermatophytes or ringworm fungi were cultivated in honey broth for 10 days until a luxuriant mycelium was formed. The mycelium and spores were triturated in a mortar with sterile saline and one drop of the suspension added to the glucose broth tubes. In the case of *Coccidioides immitis,* broth was added to cover a honey agar slant culture and, after repeated pipettings to free the culture, 2 drops of the broth were added to each of the tubes of glucose broth containing the dilutions of protoanemonin. The last 3 fungi tested—*Allescheria boydii* (*Monosporium apiospermum*), an unidentified mold from a box of strawberries, and another from an old orange—were inoculated into glucose broth by simply touching the mycelial growth with a platinum loop and transferring it to the test media. When the growth in the control tubes was abundant, the amount of protoanemonin required to give inhibition of growth of the fungi was noted. The time of incubation varied with the different species (Table III).

These experiments show that protoanemonin inhibits the growth of fungi, which proved to be as sensitive as the bacteria. The inhibit-

TABLE III.
Maximum Dilution of Protoanemonin Preventing the Visible Growth of a Number of Fungi Grown in Glucose Broth.*

Organism	Dilution of protoanemonin, × 1000	Time of reading, days
Saccharomyces cerevisiæ	50-166	2
Candida albicans (2 strains)	100-200	2
Cryptococcus neoformans (4 strains)	83-300	2
Trichophyton mentagrophyles (gypseum)	125	5
Microsporium canis	125	5
Microsporium audouini	62-83	5
Trichophyton purpureum	83	5
Coccidioides immitis	125	2 ?
Allescheria boydii	166	5
Unidentified mold from strawberry plant	62	1
Unidentified mold from orange	62	2

* See footnote Table I.

ing concentration of protoanemonin varied from 1-50,000, in the case of *Saccharomyces cerevisiae,* to 1-300,000 for *Cryptococcus neoformans.*

Methods and results with protozoa. Tetrahymena geleii[5] and *Trypanosome gambiense* were two protozoa tested for their sensitivity to protoanemonin. Four-day cultures of *Tetrahymena,* grown in 2% bactopeptone or proteose peptone at room temperature (26°-28°C), were added in 0.5 cc amounts of 4.5 cc of peptone broth containing dilutions of protoanemonin varying from 1-200,000 to 1-500,000. In the tube containing 1-200,000 dilution of protoanemonin only a very occasional sluggish, round, small organism with many fine granules might be seen after one day, while in 2 days none were discernible. The 1-300,000 dilution produced a markedly modified growth but did not usually kill. At the end of 2 days the organisms were still small and far less active. Morphologically the stoma was hard to distinguish and the granules appeared smaller and more numerous than in the control organisms. The protozoa in the tubes containing protoanemonin diluted 1-400,000 and 1-500,000 showed increasingly less variation from the normal growth, which was distinguished by the appearance of many actively mobile organisms with several undergoing fission in each field.

The sensitivity of *Trypanosome gambiense* was investigated by inoculating 2 drops of citrated infected guinea pig blood into 1 or 2 cc amounts of Weinman's semi-solid cell free medium[6] containing different concentrations of protoanemonin. The cultures were incubated at room temperature (26°-28°C). The trypanosomes in the control tubes had grown in 5 days after inoculation. No trypanosomes were demonstrable in dilutions of protoanemonin 1-200,000 at the end of 5 days, whereas active organisms were present in the 1-400,000 dilutions which appeared similar to those organisms in the control tubes (Table IV).

In other tests the inoculum was a drop from a 9-day culture of *T. gambiense.* Growth of trypanosomes was absent in the 1-1,600,000 dilution after 5 days of observation.

Methods and results with bacteriophages. An investigation was undertaken to determine the effects of protoanemonin on coli and on staphylococcus bacteriophage. Two sets of tubes containing protoanemonin diluted 1-2000 in bacteriophage were prepared. One set was incubated at 37° C for 1½ hours, the other set remained at room temperature over night. Serial dilutions in broth from 10^{-1} to 10^{-9} were then seeded with their respective organisms. In the controls distilled water was substituted for protoanemonin. The bacteriophage titer was the same in all the tests, the ones in which the bacteriophages were first incubated with the antibiotic and those in which distilled water was substituted for pro-

[5] Furgason, W. H., *Arch. Protistenkunde,* 1940, **94**, 224.

[6] Weinman, D., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 82.

TABLE IV.
Inhibition of Growth of Two Protozoa by Varying Dilutions of Protoanemonin.

Organism	Dilution of Protoanemonin						Controls	Media used	Time of reading, days
	200,000	300,000	400,000	500,000	1,600,000	3,200,000			
Tetrahymena geleii (3 strains)	0	+	+++	+++			++++	2% proteose peptone or bactopeptone	2
Tryp. gambiense guinea pig blood	0		++++				++++	Weinman's	5
Tryp. gambiense culture					0	++++	++++	"	5

toanemonin. The experiments, thus, failed to show any inhibitory effect of protoanemonin on either coli or staphylococcus bacteriophage.

Methods and results of testing influenza virus grown in chick embryos. In order to study the action of protoanemonin on the growth of influenza virus in fertile eggs, it was necessary, first, to determine the amount of protoanemonin which could be tolerated by the chick embryo. The tests showed that 0.2 cc of a 1-1000, 1-2000 or 1-4000 dilution of protoanemonin was not toxic when injected 2 days in succession in 11- or 12-day-old fertile eggs and the latter 2 concentrations were harmless to 10-day-old eggs.

The treatment of influenza infected eggs with protoanemonin in dilutions non-injurious to the chick embryo then was attempted. The combination of 1-2000 dilution of protoanemonin and 10^{-5} or 10^{-6} dilution of influenza virus killed the embryo, while the same dilution of protoanemonin with 10^{-7} dilution of virus resulted in a viable embryo with a concentration of virus comparable to that in the control eggs. The growth and titer of the virus in the allantoic fluid was determined by an Hirst[7] agglutination test. The eggs treated with 1-4000 protoanemonin and infected with the same dilutions of virus survived, and it was evident that there was a multiplication of the virus as demonstrated by the agglutination titer. These experiments would indicate that a combination of protoanemonin, non-toxic by itself, and influenza virus in adequate concentration is lethal to the embryo. Furthermore, where conditions are such that the embryo survives there is no evidence of inhibition by protoanemonin of the growth of the virus.

Tissue culture tests. The effect of protoanemonin on tissue cells was tested through the courtesy of Dr. Mary Parshley. Whole thicknesses of chicken skin were planted in chicken plasma diluted one-third with two solutions, one of which was optimal for fibroblasts and the other optimal for epithelial cells. These two solutions contained protoanemonin in concentrations of 1-1 million and 1-5 million. When compared with the

[7] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

growth of the controls, it appeared that protoanemonin was toxic to both fibroblasts and epithelial cells in 1-1 million dilution and inhibitory to growth in the 1-5 million dilution. However, it seemed to be less inhibitory for both cells when they were cultivated in the solution which favored the growth of epithelial cells.

Discussion. Protoanemonin is of interest because of its wide range of activity. *In vitro* it inhibits the growth not only of Gram-positive and Gram-negative bacteria but also fungi, two protozoa and fowl epithelial and fibroblastic tissue cells.

The action of protoanemonin on bacteria, fungi and the tetrahymena involves two types of effect, an inhibition of the growth of the microorganisms and an actual killing of the organisms. Only the former effect has been considered in the data presented here. In most cases subcultures from the tubes containing the greatest dilution of protoanemonin inhibiting growth, as reported in the tables, would demonstrate the presence of viable organisms. Indeed, this frequently might be demonstrated by the simple procedure of further incubation of the original tubes. Greater concentrations of the protoanemonin, however, actually kill the organisms. This phase of the action of protoanemonin will be described at a later time.

Another factor under investigation which may influence the action of protoanemonin is the composition of the medium. It may be observed that the bacteria tested for sensitivity to protoanemonin in both meat infusion broth and casein hydrolysate broth were inhibited by greater dilutions of protoanemonin in the latter medium. This influence of medium on the sensitivity of an organism to protoanemonin was apparently unrelated to the relative growth of the organisms in the two broths. *Staphylococcus oxford* H. *Vibrio cholerae* and *Pseudomonas aeruginosa* grew better in the infusion broth, while *Escherichia coli* and *Corynebacterium xerosis* grew as well or better in the casein hydrolysate broth. The casein hydrolysate medium was employed because it is apparent, as seen in the tables, that the greatest dilution of protoanemonin causing inhibition of growth of a given organism sometimes varied considerably in different tests. It was thought that the introduction of a semi-synthetic medium, more uniform in its composition than meat infusion broth, might prevent this fluctuation. It has continued, however, even when casein hydrolysate broth was used. The explanation for this is not at present available.

Certain mammalian bloods, when added to culture media, cause a decrease in sensitivity to protoanemonin. For example, guinea pig blood is highly inhibitory. This may contribute to the difference in the titer of activity of protoanemonin against *Trypanosome gambiense* in tubes inoculated with infected guinea pig blood and those inoculated from culture media. There also is the possibility that the diverse forms of trypanosomes found under the two conditions of growth contribute to the variation in sensitivity.

The method of testing the sensitivity of the bacteriophages was such that protoanemonin was in contact only with the resting bacteriophage. Experiments to be reported indicate that protoanemonin is relatively harmless to resting bacteria and is antagonistic mainly during the period of active growth. It is therefore possible that the method was not suitable to indicate the effect of this agent on bacteriophage. The antibiotic nature of protoanemonin makes it difficult to evaluate when the organism tested requires a viable and actively growing substrate. Thus the failure to demonstrate inhibition of the influenza virus may have been due to the fact that such small amounts of protoanemonin were tolerated by the chick embryo that the effective concentration of protoanemonin for the virus was not achieved.

Summary. 1. Protoanemonin inhibited the growth of all the aerobic and anaerobic bacteria, the fungi and the protozoa tested. 2. The maximum dilution of protoanemonin which was effective against the bacteria and fungi varied from 1-6000 to 1-300,000. 3. The anti-bacterial activity of protoanemonin was independent of the acidity of the medium, the size of the inoculum and the age of the culture, within the limits tested. 4. The two

protozoa were prevented from growing in dilutions of protoanemonin ranging from 1-200,000 to 1-1,600,000. 5. No inhibition by protoanemonin of the growth of the two bacteriophages and the influenza virus was demonstrable by the techniques employed. 6. A dilution of 1-1,000,000 protoanemonin was toxic for chicken tissue culture epithelial and fibroblastic cells.

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Determination of O_2 Capacity on 39.3 Cubic Millimeters of Blood.

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In a recent study of bone marrow blood gases[1] in which the use of minimal sample volumes was imperative, estimation of O_2 capacity was necessary. The current procedure used for such determinations involves aeration of blood after which the O_2 content is determined. This technique has been used by Roughton and Scholander[2] and by Lilienthal and Riley.[3] Preliminary aeration was carried out in a separate vessel with 0.5 or more cc of blood although only 39.3 cmm were used for the O_2 determination. When blood is aerated in a flask or syringe, a certain amount of plasma is lost by evaporation as well as by adherence to the sides of the vessel. Should quantities of blood smaller than 0.5 cc be used, concentration of the red cells becomes greater. To avoid the errors inherent in separate aeration and to reduce the amount of blood required, the micro method of Roughton and Scholander has been modified so that the O_2 capacity can be determined on a total blood sample of only 39.3 cmm.

Apparatus. The Roughton-Scholander syringe and pipette (Roughton and Scholander[2]) are employed. A second mark (designated "upper mark") is scratched on the syringe cup at a distance above the existing one such that the volume of the cup as measured between the two marks is approximately 25 cmm. Since the diameter of most cups is 2.5 mm, the upper mark may be made 5 mm above the first. In the estimation of O_2 capacities exceeding 16 vol % the syringe with the 50 unit capillary is necessary.

Reagents. In addition to those listed by Roughton and Scholander 0.9% NaCl is required.

Sampling blood. From a finger prick or a needle inserted in a vessel, blood is sucked directly into the 39.3 cmm pipette which has been previously flushed with anti-coagulant solution (heparin) and dried in a current of air.

Procedure. 1. The syringe is flushed 3 times with separate portions of saline, emptied, and the cup filled to the lower mark with saline.

2. The pipette is filled to its mark with blood; its tip is passed carefully into the cup containing saline and pressed firmly against the bottom. 3. Blood is sucked into the syringe by a smooth withdrawal of the plunger. Once the pipette is emptied it is quickly removed and the saline in the cup is drawn in after the blood.

4. The blood-saline mixture is lowered far enough into the syringe to permit entry of an air volume of approximately 1.0 cc.

5. The syringe is then rotated for 5 minutes on its long axis with an occasional rotation at right angles to spread a small layer of blood over the inside of the syringe and thus ensure

1 Grant, W. C., and Root, W. S., *Fed. Proc.*, 1947, **6**, 114.

2 Roughton, F. J. W., and Scholander, P. F., *J. Biol. Chem.*, 1943, **148**, 541.

3 Lilienthal, J. L., Jr., and Riley, R. L., *J. Clin. Invest.*, 1944, **23**, 904.

TABLE I.
Comparison of Oxygen Capacities of Blood by Sendroy Method (1.0 cc) and Micromethod (39.3 cmm).

Sendroy, Vol. %	Micro, Vol. %	Difference, Vol. %	Hct. (Van Allen), %
20.3	20.2	—0.1	45.9
19.6	19.8	+0.2	44.5
15.2	15.1	—0.1	35.9
16.7	16.8	+0.1	37.3
17.1	17.2	+0.1	40.8
14.5	14.2	—0.3	35.9
12.5	12.8	+0.3	30.8
10.5	10.4	—0.1	24.1
15.8	15.7	—0.1	38.0

uniform exposure of blood to the air. Once a minute during this procedure the air is renewed by alternately running the blood up to the capillary and lowering to the 1.0 cc mark. In certain syringes the formation of transverse blood films makes the expulsion of air without loss of blood difficult. The addition of 2 capillary divisions of caprylic alcohol alleviates this condition.

6. Air is expelled and blood-saline pushed up to the bottom of the cup. The cup is filled to the upper mark with ferricyanide solution, and the solution is then lowered to the bottom of the cup.

7. Two divisions of caprylic alcohol are drawn into the top of the capillary and the cup emptied.

8. Acetate buffer is added to the upper mark and the original Roughton-Scholander[2] (page 545) procedure for O_2 content is then followed.

Blank determination. To obtain a blank value for reagents as well as for physically dissolved oxygen of the blood, an analysis is performed exactly as indicated above; but instead of delivering the usual 39.3 cmm of blood, 43.3 cmm of 0.9% NaCl is substituted. For this purpose a mark indicating 43.3 cmm volume, or 110% of the usual 39.3 cmm volume, is scratched on the pipette. Sendroy *et al.*[4] have shown that for practical purposes 1.1 cc of aerated saline solution contains the same volume of physically dissolved O_2 as 1.0 cc of blood.

Calculations. Calculation of results follows that described by Roughton and Scholander.[2]

Aeration of blood. Quite unexpectedly complete saturation of the blood mixture is obtained in only 5 minutes. This is accomplished by the vigorous rotation of the blood with air which is expelled and replaced with fresh air at intervals of a minute, as described above. The ratio of total air to liquid volume is approximately 100 to 1. That a longer time of aeration does not increase the O_2 content was demonstrated by noting that, on the same blood, two determinations after 5 minutes aeration showed O_2 capacities of 17.0 and 16.8 vol % and two others after 10 minutes of aeration, 16.9 and 16.9 vol %.

Accuracy of method. The O_2 capacity as determined on 1.0 cc of blood with the standard method of Sendroy[5] was compared with that measured upon 39.3 cmm of the same blood with the micromethod. These analyses were performed by 2 individuals, one using the Sendroy method and the other the micromethod. The results obtained on 6 dogs are shown in Table I. Hematocrit values measured with Van Allen[6] tubes are included for reference. The agreement between the 2 procedures was good, the maximum difference being 0.3 vol %.

Successive measurements performed on the same sample by the micromethod yielded variations of similar magnitude as demonstrated in the following 2 samples: (a) 15.5, 15.6 vol % and (b) 11.2, 11.4, 11.2 vol %.

Discussion. The Roughton-Scholander micromethod for the measurement of the O_2 content of blood has been modified so that O_2 capacity can be determined on a total blood sample of 39.3 cmm. Since the blood is both aerated and analyzed in the same apparatus the transfer from aeration vessel to Roughton-Scholander syringe is avoided. The elimination of this step which is required when aeration is carried out in a separate vessel avoids the possibility of analyzing blood which contains a concentration of red cells unlike that obtained from the subject.

The method permits the estimation of O_2

[4] Sendroy, J., Jr., Dillon, R. T., and Van Slyke, D. D., *J. Biol. Chem.*, 1934, **105**, 597.

[5] Sendroy, J., Jr., *J. Biol. Chem.*, 1931, **91**, 307.

[6] Van Allen, C. M., *J. Lab. and Clin. Med.*, 1925, **10**, 1027.

capacity on blood obtained from a finger prick. It should prove useful where measurements are needed on small animals such as mice or rats or under conditions where venipuncture is difficult or undesirable, as in premature infants.

Summary. The Roughton-Scholander microgasometric procedure has been modified to provide a simple and accurate micromethod for the determination of the O_2 capacity of blood. Only 39.3 cmm are required and an analysis may be completed in 10 to 12 minutes. O_2 capacities obtained by the micromethod agree well with those measured by the Sendroy macromethod, the average difference being ± 0.16 vol %.

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Renal Excretion of Mannitol.*

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The evidence that the clearance of inulin in man is a measure of the rate of glomerular filtration, although necessarily indirect, seems fairly conclusive.[1] Other studies by Smith and his colleagues[2] have suggested that a number of hexitols (including mannitol) are excreted solely by glomerular filtration. Finally, Gilman has reported[3] that the clearance of thiosulfate is also a measure of the filtration rate.

Recently, a patient was observed in this laboratory whose inulin clearance was found to be 144 ml per minute, but whose mannitol clearance, 3 months later, was 94 ml per minute. Since this patient had no demonstrable kidney disorder and since there was no reason to expect any significant change in renal function during the elapsed period, it appeared likely that inulin and mannitol clearances are not always identical. Further observations on this point, therefore, were undertaken and form the basis of this report.

Some support for the belief that the mannitol clearance is less than that of inulin is obtained from published reports. The most extensive series of observations on the inulin clearance in normals is that of Smith and his colleagues[4] who found the average value of 131 ml per minute with a standard deviation of 21.5 ml per minute in 67 males, and an average of 117, with a standard deviation of 15.6, in 21 females. The findings of most other investigators are in line with those of Smith and are summarized in Table I. Also included are previously unpublished data from this laboratory. The average inulin clearance for the entire group of 133 males is 128 ml per minute. In contrast, the average mannitol clearance in 52 males is 116 ml per minute. The difference between the mean mannitol and inulin clearances among the male patients is statistically significant. There is little difference between the mannitol and inulin clearances among the female patients.

There are but few published data on direct comparisons between mannitol and inulin

* The work described in this paper was supported by a grant from the Carnegie Corporation of New York.

1 Smith, H. W., *Physiology of the Kidney,* Oxford University Press, New York, 1937.

2 Smith, W. W., Finkelstein, N., and Smith, H. W., *J. Biol. Chem.,* 1940, **135**, 231.

3 Newman, E. V., Gilman, A., and Philips, F. S., *Bull. Johns Hopkins Hosp.,* 1946, **79**, 229.

4 Smith, H. W., *Lectures on the Kidney,* University of Kansas, Extension Division, 1943.

TABLE I.
Summary of Inulin and Mannitol Clearances in Normal Human Subjects Reported from Various Laboratories. All values are corrected to a surface area of 1.73 sq. m.

Author	Male			Female		
	No. of subjects	Mean clearance ml/min.	Standard deviation	No. of subjects	Mean clearance ml/min.	Standard deviation
			Inulin.			
Smith[4]	67	131	21.5	21	117	15.6
This laboratory*	26	126	17.1	7	111	16.5
Friedman[6,7]	16	129	30.6	5	126	14.0
Bradley[12]	8	114	15.0	6	128	19.5
Foa[8]	7	117	30.8			
Miller[9]	5	115	16.4			
Talbott[10]	4	150	35.8			
Total group	133	128	23.5	39	119	16.4
Standard error of the mean: 2.04.						
			Mannitol.			
This laboratory*	23	111	17.8	2	108	
Bradley[12]	10	141	43.0	23	124	18.9
Newman†[11]	9	103	15.6			
Klopp[13]	6	117	23.8			
Lauson‡[14]	4	107	25.5	1	125	
Total group	52	116	28.6	26	123	24.5
Standard error of the mean: 3.97.						

Standard error of the difference of the means for males: 4.46, t = 2.69. Probability of such a difference occurring by chance, less than 0.007.

* Includes published[5] and unpublished data.

† Estimated from Fig. 4 of the paper by Newman *et al.*[11]

‡ Data derived from patients 17-76 days after recovery from shock.

clearances. The average ratio of mannitol to inulin clearance in 8 observations on 6 patients reported in the original paper on mannitol[2] was 0.99 with a range of 0.96 to 1.03. One of these patients had hypertension, 2 were normal ante-partum females, while the remainder had preeclampsia. Eight comparisons were reported[5] among 7 patients with various degrees of renal functional impairment due to glomerulonephritis. The mean ratio was 0.96, with a range of 0.92 to 1.03. Lauson and his colleagues reported[14] a mean mannitol to inulin clearance ratio of 0.90 among 5 patients suffering from shock. The authors state, however, that they were experiencing minor technical difficulties with the inulin method and that ". . . it appears likely that the average difference of 10% represents a systematic technical error rather than a true difference in clearance." It is to be noted that the majority of the reported comparisons between mannitol and inulin clearances were performed in patients who either did have or probably had some renal abnormality. Three comparisons between the mannitol and creatinine clearances were performed in 2 dogs and are reported in the original paper on mannitol.[2] In one dog, the ratio was 0.98, but both experiments in the other animal yielded a ratio of 0.92.

[5] Earle, D. P., Taggart, J. V., and Shannon, J. A., *J. Clin. Invest.*, 1944, **23**, 119.

[6] Friedman, M., Selzer, A., and Rosenblum, H., *J. Clin. Invest.*, 1941, **20**, 107.

[7] Friedman, M., Selzer, A., Sugarman, J., and Sokolow, M., *Am. J. Med. Sc.*, 1942, **204**, 22.

[8] Foa, P. P., Woods, W. W., Peet, M. M., and Foa, N. L., *Arch. Int. Med.*, 1942, **69**, 822.

[9] Miller, B. F., Alving, A. S., and Rubin, J., *J. Clin. Invest.*, 1940, **19**, 89.

[10] Talbott, J. H., Pecora, L. J., Melville, R. S., and Consolazio, W. V., *J. Clin. Invest.*, 1942, **21**, 107.

[11] Newman, E. V., Bordley, J., and Winternitz, J., *Bull. Johns Hopkins Hosp.*, 1944, **75**, 253.

[12] Bradley, S. E., personal communication.

[13] Klopp, C., Young, N. F., and Taylor, H. C., *J. Clin. Invest.*, 1945, **24**, 117, 189.

[14] Lauson, H. D., Bradley, S. E., and Cournand, A., *J. Clin. Invest.*, 1944, **23**, 381.

Methods. All inulin and mannitol† clearances reported in this paper for man were performed in patients with no demonstrable renal abnormalities, and represent the average of at least 3 consecutive periods. Urine was collected by a many-eyed catheter, with bladder rinses with sterile water, followed by an injection of air. Creatinine clearance was used as the standard of comparison in similar studies in the dog.

Chemical. Inulin was measured by Harrison's modification[15] of the Alving method.[16] The urines as well as the plasmas were yeasted so that any possible adsorption of inulin on yeast would not constitute an error in the determination of the inulin clearance. Mannitol was measured in some instances by a modification[5] of Smith's technique[17] and, in all experiments, by the chromotropic acid method of Corcoran and Page.[18] Creatinine was estimated by a modification of the Folin procedure.[19]

Since much of this study was based on simultaneous mannitol and inulin clearances, it was important to investigate any possible interference of one substance with the chemical determination of the other. In these experiments, the concentration of substance run through its specific analysis was in the range utilized in the clearance studies, while the possible interfering substance was added both at the concentration used in the clearances and in excess. Each study was done in duplicate. The results of the examination of the inulin procedure will be presented in detail.

One mg percent and 10 mg % solutions of mannitol reacted with diphenylamine reagent as in the inulin procedure gave readings identical to water treated in the same manner. A 1 mg % standard inulin solution gave the same result when run through the reaction above or in the presence of 1 to 10 mg % mannitol. Five, 10, and 15 mg % inulin standards, yeasted and then run through the diphenylamine reaction, resulted in a mean recovery of 99.5% compared to aqueous non-yeasted solutions, while the mean recovery for a similar series of inulin standards was 99.6% in the presence of 150 mg % mannitol. The mean recovery of 10, 15, and 25 mg % inulin solutions when added to normal plasma was 98.8% and when added to plasma containing 100 mg % mannitol, was 97.6%. The addition of 150 mg % mannitol to normal plasma had no effect on the apparent inulin blank, nor did 150 mg % mannitol solution change the apparent concentration of inulin in the plasma of a patient receiving this material. Finally, the presence or absence of mannitol in urine containing inulin made no difference in the result obtained with the inulin reaction. The urines in these studies were yeasted.

Similarly, the presence of inulin did not alter the analysis of mannitol by the chromotropic acid method, either in aqueous solutions, in plasma, or in urine, the recovery of mannitol in all these circumstances ranging from 99 to 101% whether or not inulin was present. Inulin also did not alter the apparent plasma blank for mannitol. Inulin did not interfere with the analysis of mannitol by the titration method, either in aqueous solutions or after yeasting.

Results. Simultaneous inulin and mannitol clearances were performed as 9 separate experiments in 8 normal subjects. In each instance, the mannitol clearance was less than that of inulin, the mannitol to inulin clearance ratio ranging from 0.79 to 0.96. The mean inulin clearance for the group was 125 ml per minute, the mean mannitol clearance was 109, yielding a mean clearance ratio of 0.87. These data are summarized in Table II. Mannitol was measured by Smith's titration method as well as by the chromotropic acid method

† The mannitol used in these studies was obtained through the courtesy of Dr. Karl Beyer of Sharp & Dohme, Inc.

15 Harrison, H. E., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 111.

16 Alving, A. S., Rubin, J., and Miller, B. F., *J. Biol. Chem.*, 1939, **127**, 609.

17 Goldring, W., and Chasis, H., *Hypertension and Hypertensive Disease*, Commonwealth Press, 1944.

18 Corcoran, A. C., and Page, I. H., *Fed. Proc.*, 1946, **5**, 130.

19 Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, **38**, 81.

TABLE II.
Comparison of Inulin and Mannitol Clearances in Males.
All values represent the average of 3 or more consecutive periods and are corrected to a surface area of 1.73 sq. m.

Patient	Age	Date	Inulin clearance ml/min	Mannitol clearance ml/min	Clearance ratio mannitol/ inulin	Control inulin clearance ml/min	Decrease after mannitol %	Control mannitol clearance ml/min	Decrease after inulin %
Las	46	1/2	142*						
		2/26	141*						
		4/11	139	118	0.85	158	12.0		
Sol	42	4/9	105	101	0.96	124	14.8		
McC	31	4/18	155	140	0.90	134	11.5		
War	34	3/26	138	109	0.79				
		4/15	116	110	0.95	122	6.1		
Alv.	46	4/23	126	112	0.89			122	7.9
Vad	39	4/28	156	123	0.79			134	8.4
		5/15	144*						
Mor	40	3/24	116*						
		4/7	96.5	84.8	0.87				
Sch	48	3/19	96.0	94.0	0.88				
Avg			125	109	0.87	135	11.1		

* Not included in average.

in 2 of the experiments. The results were identical for both methods. In 4 of the studies shown in Table II, control inulin clearances were measured prior to the infusion of mannitol. The inulin clearance was lower after mannitol in each instance, the average depression being 11.1%. The reverse experiment, wherein control mannitol clearances were obtained before the administration of inulin, resulted in smaller and transient depressions in mannitol clearance. The average decreases in mannitol clearance were 7 and 8%, but in each instance the mannitol clearance had almost returned to control values within one hour after the mannitol injection. In several patients (Las, Mor, and Vad, Table II), inulin clearances alone were performed on another date. In each instance, the inulin clearance done separately more closely approximated the inulin than the mannitol value when the two were done simultaneously.

The creatinine clearance is generally believed to be a measure of glomerular filtration rate in the dog. For this reason, mannitol and inulin clearances were compared separately to that of creatinine in 3 unanesthetized female dogs. These data are summarized in Table III. The mean inulin to creatinine clearance ratio was 1.01, while the mannitol to creatinine clearance ratio in the same 3 dogs was 0.88.

Although metabolic alteration of a compound does not preclude its use as a measure of glomerular filtration rate by urine collection techniques, the application of the constant infusion technique[20] requires that there be no metabolic alteration of the substance. If the renal clearance of a substance, as measured by urine collections, is less than the clearance as measured by the infusion technique, it may be concluded that some of the substance is metabolized. The ratios of the clearances of mannitol as measured by the two techniques ranged from 0.75 to 1.00, with an average of 0.88. The mean urine to infusion pump clearance for inulin on 31 subjects yielded a ratio of 1.02.[21] In 3 normal subjects, 80, 85, and 90% of administered mannitol was recovered from the urine collected for 24 hours after the dose. Smith and his co-workers[2] likewise found 81 and 89% recovery of mannitol during the 10½-hour period after the dose, but in similar experiments with inulin, recovered 95 and 97% in the same 2 patients.

Discussion. The mannitol clearance has been found to be consistently lower than the simultaneously measured inulin clearance in 9 patients with no demonstrable renal dis-

[20] Earle, D. P., and Berliner, R. W., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 262.

[21] Unpublished data.

TABLE III.
Comparison of Inulin and Mannitol to Creatinine Clearances in Dogs.
All values represent the average of 3 or more consecutive periods.

	Clearances, ml/min			Ratios	
Dog No.	Creatinine	Inulin	Mannitol	Inulin/creatinine	Mannitol/creatinine
1	52.0	53.0		1.02	
	66.2		59.5		0.90
2	51.5	52.2		1.01	
	39.7		33.9		0.85
3	69.3	69.2		1.00	
	62.4		55.8		0.89
			Avg	1.01	0.88

orders. In addition, the mannitol clearance is less than the simultaneous creatinine clearance in the dog, while the inulin clearance has been consistently equal to the creatinine clearance. Recently, Smith and his co-workers[22] have found that mannitol clearances are less than creatinine in the dog. Corcoran[23] has found an average mannitol/inulin clearance ratio of 0.902 with a standard error of 0.017 for 42 observations in dogs and human beings. The mannitol/creatinine clearance ratio in the dogs averaged 0.872 with a standard error of 0.013, and the average mannitol/thiosulfate clearance ratio in dogs was 0.89. Hoobler[24] observed an average mannitol/-thiosulfate clearance ratio of 0.89 with a standard deviation of 0.07 in 52 observations in 14 patients.

It also appears that the administration of mannitol by intravenous infusion results in a small but significant decrease in the inulin clearance and presumably in the glomerular filtration rate. This action is not necessarily due to the mannitol itself, but may be the result of other substances contained in the mannitol preparation and is presumably due to alterations in the glomerular hemodynamics.

The evidence summarized by Smith[1] for the belief that inulin is a measure of glomerular filtration rate in man is very strong. This belief is strengthened by the identity between inulin and creatinine clearances in the dog, it being generally believed that creatinine clearance is a measure of filtration rate in this species. For these reasons, it seems probable that the discrepancies between mannitol and inulin clearances are due to reabsorption of a small proportion of filtered mannitol by the renal tubules.

It appears likely, therefore, that mannitol cannot be used as a precise measure of glomerular filtration rate in man. Since some mannitol undergoes metabolic alteration, it certainly cannot be utilized for the measurement of filtration rate by the infusion pump technique. It should be noted that mannitol is an excellent osmotic diuretic, and that although thiosulfate appears to be a measure of filtration rate, it in itself is an electrolyte and in its excretion must carry with it its equivalent cation. For these reasons, neither mannitol nor thiosulfate is ideal for the measurement of filtration rate during the course of experiments directed toward the study of water or electrolyte excretion. Inulin, therefore, remains the substance of choice for the measurement of glomerular filtration rate in man under the widest variety of circumstances.

Conclusions. 1. The renal clearance of mannitol is frequently less than the simultaneous clearance of inulin in man. 2. The intravenous injection of mannitol depresses the inulin clearance in man. 3. The clearance of inulin is equal to that of creatinine in the dog, but the mannitol clearance is usually less than that of creatinine. 4. Mannitol undergoes metabolic alteration in man, and therefore, cannot be utilized in the infusion pump technique for measuring the rate of glomerular filtration. 5. Inulin appears to be the most generally useful substance for the measurement of glomerular filtration in man.

22 Brode, J., *et al.*, to be published.
23 Corcoran, A. C., personal communication.
24 Hoobler, S. W., personal communication.

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Dental Caries in the Cotton Rat IX. Effect of Milk Rations*

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Investigations in this laboratory on the experimental production of dental caries in the cotton rat showed that the molars of this animal were highly susceptible to decay when the rat was fed a dry ration high in fermentable sugar.[1,2] In contrast to this, animals fed on a diet consisting of liquid whole milk were found to be completely free of caries.[3] The consumption of a dry ration approximating milk solids in composition resulted in a low incidence and extent of carious lesions, although protection was not complete, as it was in the case of the liquid milk. The protection afforded by milk is as great as, or greater than, that found with any other ration, and further study of this effect therefore seemed important. Two approaches were made: (1) fermentable sugars known to be highly cariogenic when fed as the carbohydrate portion of dry rations were added to the liquid milk diet; and (2) supplements of milk were given to animals being fed the cariogenic control ration.

Experimental. The cotton rats used in these experiments were raised in our own stock colony and placed on experiment at weaning (20-25 g). Animals from each litter were distributed as equally as possible among the control and experimental groups, since susceptibility to dental caries has been found to vary among different litters.[4] The animals were kept on experiment for 14 weeks, during which time their weights were recorded at bi-weekly intervals. At the end of the experimental period, the animals were killed, and the incidence and extent of carious lesions in each animal were evaluated by the method of Shaw and his associates.[1,5]

The control animals were fed a sucrose ration (802) previously found to be highly cariogenic.† The liquid whole milk ration was fortified with minerals (iron, copper and manganese) and with 1:20 liver extract as described by Schweigert and co-workers.[3] The carbohydrate (sucrose, glucose or dextri-maltose) was added to the milk at a 5% or 10% level. This, together with liver extract, was homogenized into the milk in a Waring blendor. These diets were fed *ad libitum.* In one group milk was fed in addition to the 802 ration. In this case the milk was fed *ad libitum,* but the intake of ration 802 was limited to 63% of that of the controls (802 ration only) in order to induce greater consumption of the milk. The milk consumed constituted roughly one-third of

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1 Shaw, J. H., Schweigert, B. S., McIntire, J. M., Elvehjem, C. A., and Phillips, P. H., *J. Nutr.,* 1944, **28**, 333.

2 Schweigert, B. S., Shaw, J. H., Phillips, P. H., and Elvehjem, C. A., *J. Nutr.,* 1945, **29**, 405.

3 Schweigert, B. S., Shaw, J. H., Zepplin, M., and Elvehjem, C. A., *J. Nutr.,* 1946, **31**, 439.

4 Schweigert, B. S., Shaw, J. H., Elvehjem, C. A., and Phillips, P. H., Proc. Soc. Exp. Biol. and Med., 1945, **59**, 44.

5 Shaw, J. H., Schweigert, B. S., Elvehjem, C. A., and Phillips, P. H., *J. Dent. Res.,* 1944, **23**, 417.

† This ration consisted of 67% sucrose, 24% casein, 5% corn oil and 4% salts IV. Four per cent of 1:20 liver extract was added at the expense of the entire ration. Adequate quantities of the B vitamins[6] were added to the ration, and each rat received one drop of halibut liver oil per week.

6 McIntire, J. M., Schweigert, B. S., and Elvehjem, C. A., *J. Nutr.,* 1944, **27**, 1.

TABLE I.
Effect of Various Milk Rations on Growth Rate and on Incidence and Extent of Carious Lesions in the Cotton Rat.

Ration	No. of pairs	Avg weekly gain 6 wks	Avg weekly gain 14 wks	Avg incidence	Avg extent
802*	15	10.1	7.2	29	75+
milk		7.3	5.3	0	0†
802	3	8.4	5.5	30	76+
milk + 5% sucrose		6.6	4.8	0	0
802*	11	9.5	6.5	32	82+
milk + 10% sucrose		7.4	5.5	4	7+
802	3	11.6	7.8	34	109+
milk + 5% dextrimaltose		10.3	7.2	1	1+
802	7	11.1	8.0	24	50+
milk + 10% dextrimaltose		8.5	7.4	0	0
802*	13	9.0	6.7	27	68+
milk + 10% glucose		7.3	5.8	6	10+
802	9	9.5	7.8	31	78+
802 + milk		6.7	7.7	14	25+

* These groups included one animal which died and was evaluated at 12 weeks instead of at 14 weeks.

† One of these 15 animals had a 1 — 1+ lesion.

the daily caloric intake of the animals in this group.

Results. In Table I the data from the animals on each experimental ration are compared with those from their respective litter-mate controls. The control and experimental animals from each litter were paired so that averages represented equal numbers of animals.

Growth rates were calculated as average weekly gain for the first 6 weeks and for the total 14 weeks on experiment. Since the two sexes have been found to grow at different rates, all figures were calculated as growth of males in order to make them comparable. This was formerly done by increasing the values for the females by 25%;[2] but analysis of a larger volume of data now at our disposal has shown that more accurate factors are 20% at 6 weeks and 15% for the total 14 weeks. Accordingly, the gains of females were increased by these percentages for the respective periods. From the data it is evident that the animals fed on milk alone grew less than those receiving the control ration (802). Growth was somewhat better when supplemental carbohydrates were added to the milk, although it was still below that of the control animals. The animals fed ration 802 plus milk grew slowly during the first weeks of the experiment, but the average weekly gain for the total 14 weeks was very nearly the same as that for the controls receiving only the dry ration.

Table I also gives the data on incidence and extent of carious lesions. The comparison of average scores for the experimental animals with those for litter-mate controls receiving the cariogenic ration (802) indicates the degree of protection afforded by each ration. The scores of animals on the milk diets were low in comparison with the high incidence and extent of lesions found in animals fed the control ration. In agreement with previous findings, animals fed only liquid milk were free of caries (except one animal, which developed a small lesion—1+). The caries indices of animals receiving milk with added carbohydrate varied considerably, ranging from zero to less than one-fourth the average for litter-mate controls. In the case of the animals fed milk plus ration 802, the milk appears to have reduced the caries scores by more than 50%, the

averages being 14 and 25+ for the experimental animals as compared with 31 and 78+ for the controls. Thus, the replacement of approximately one-third of the caloric intake by liquid milk resulted in a lowered caries score.

Discussion. From these data it appears that a diet of liquid whole milk is protective against dental caries in the cotton rat. Even in the presence of fairly high levels of fermentable sugars, it exerts a protective action. The protection is less, but evident, when a cariogenic dry ration is fed in addition to the milk. Since it is probable that such protection is the result of a combination of factors, it is difficult to speculate on the mechanism involved.

On a percentage basis the composition of these milk diets, even with the carbohydrate additions, resembles that of a "medium fat" ration used in earlier experiments.[3,7] In previous work all dry rations with this medium fat level have given partial protection against caries, although scores have not usually been as low as they were on these milk diets.

The kind of carbohydrate may also have been important. The carbohydrate portion of these diets was at least partially lactose, which appears from our preliminary evidence to be somewhat less cariogenic than the more fermentable sugars. The effects of the various fermentable sugars, however, seem to be similar. The caries scores on the milk plus carbohydrate rations showed considerable variation; there appeared to be no consistent trend, and overlapping of individual scores occurred. In dry rations of the medium fat level, these same sugars—sucrose, glucose and dextri-maltose—showed no significant differences in caries production.[7]

The differences in caries scores between the rats fed only a liquid diet and those receiving dry ration in addition suggests that the physical state of the ration may play a considerable role in the protective action. In the liquid diets carbohydrates were consumed in the dissolved state, and it is possible to attribute the protection exhibited to a lack of mastication or to the fact that the carbohydrates probably remained in the mouth for a comparatively short time. The lower growth rates of the animals on the liquid diets, as compared with their controls, indicate that a difference in caloric intake may also have been a contributing factor.

In this connection it is interesting to note the possible effect of the condition of the fat, which exists in relatively free form in liquid milk. Rosebury and Karshan[8] found that free oil or fat in the diet was effective in reducing the caries index in white rats. They postulated that lipids may form a mechanical coating on the tooth surface or on the particles of food, thus protecting against bacterial action, an idea also suggested by McCollum.[9]

As yet, inadequate investigation has been made of the effect of milk on the growth and activity of microorganisms associated with dental decay, and an inhibitory effect through this channel might be possible. Other conditions of the oral environment, such as pH, might also play a role. Further studies are in progress.

Summary. Data on growth and caries indices of cotton rats fed various milk rations were secured from these experiments.

The data indicate that milk is protective against dental caries in the cotton rat. Previous findings of zero scores in animals fed only liquid milk were confirmed. Animals receiving milk to which sucrose, glucose or dextri-maltose had been added exhibited low caries scores as compared with controls on a cariogenic dry ration. These sugars will produce caries when fed in dry rations. The caries indices of animals receiving approximately one-third of their caloric intake as liquid milk and the remainder as the cariogenic ration 802 were less by 50% than those of litter-mate controls not receiving milk.

7 Schweigert, B. S., Potts, E., Shaw, J. H., Zepplin, M., and Phillips, P. H., *J. Nutr.*, 1946, **32**, 405.

8 Rosebury, T., and Karshan, M., *J. Dent. Res.*, 1939, **18**, 189.

9 McCollum, E. V., Orent-Keiles, E., and Day, H. G., *The Newer Knowledge of Nutrition*, 5th ed., Macmillan, New York, 1939.

15985

Nicotinic Acid Oxidation in *Pseudomonas fluorescens.**

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In the determination of nicotinic acid in blood, Allinson[1] first employed a microorganism as the source of an enzyme which specifically destroyed nicotinic acid. The microorganism used was the NC (neutral culture) isolated from soil by Dubos and Miller.[2] Later Koser and Baird[3] made an extensive investigation of bacteria which destroy nicotinic acid during cell multiplication. They found that bacteria of the *Pseudomonas fluorescens* and *Serratia marcescens* groups grew on a synthetic medium in which nicotinic acid was the only organic compound and that they failed to grow when nicotinic acid was replaced by the isomers, isonicotinic acid and picolinic acid.

The nature and mode of action of the bacterial enzyme have not yet been elucidated. The experiments recorded below show that in the case of *Pseudomonas fluorescens* an oxidative mechanism is involved in the enzymic decomposition of nicotinic acid.

Materials and Methods. I. *Culture:* Preliminary tests with strains 1, 2, 3, 4, 12 and 30‡ showed that the latter 2 were most active in decomposing nicotinic acid. *Serratia marcescens* took 7 to 8 days to destroy as much nicotinic acid as was destroyed by *Pseudomonas fluorescens* in 36 hours. The growth of the NC organism was unsatisfactory when the medium was prepared with local tap water or artificial tap water.[4] Since *Pseudomonas fluorescens* 30 was most active and its mineral requirements were easily met by tap water or distilled water plus magnesium sulphate, it was chosen as the test organism. It was grown in media composed of: 2 g Na_2HPO_4; 1.5 g KH_2PO_4; 5 g NaCl; 0.1 g $MgSO_4$; and 2 g nicotinic acid in 1 liter distilled water. The pH was adjusted with sodium hydroxide to 6.8 to 6.9. The temperature was 27°C. The bacteria became well adapted to the medium by repeated transfer of 24 hour cultures. Stock cultures were maintained on agar slopes of nicotinic acid medium and no loss of activity was evident after 6 months. The agar cultures had a bright green color after 18 to 24 hours, which turned to reddish-brown after 48 hours.

To prepare suspensions of the *resting bacteria* transfers were made from the nicotinic acid slopes to 50 ml of medium in 250 ml Erlenmeyer flasks. The flasks were shaken for 24 hours at 25°C after which 10 ml were transferred to 500 ml of medium in a 2-liter flask, the latter being shaken again for 18 to 24 hours. The cells were then separated by centrifuging, washed twice with M/50 phosphate buffer of the pH 7.0, and finally suspended in a volume of sterile water equal to 1/20th the volume of the culture medium. This suspension was stored at 5°C for over 2 months without loss of activity.

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[1] Allinson, C. M. J., *J. Biol. Chem.*, 1943, **147**, 785.

[2] Dubos, R., and Miller, B. F., *J. Biol. Chem.*, 1937, **121**, 429.

[3] Koser, S. A., and Baird, G. R., *J. Infect. Dis.*, 1944, **75**, 250.

‡ The authors wish to express their thanks to Prof. S. A. Koser, Department of Bacteriology, University of Chicago, for the strains 12 and 30 of *Pseudomonas fluorescens* used in this work.

[4] Miller, B. F., Allinson, M. J. C., and Baker, Z., *J. Biol. Chem.*, 1939, **130**, 383.

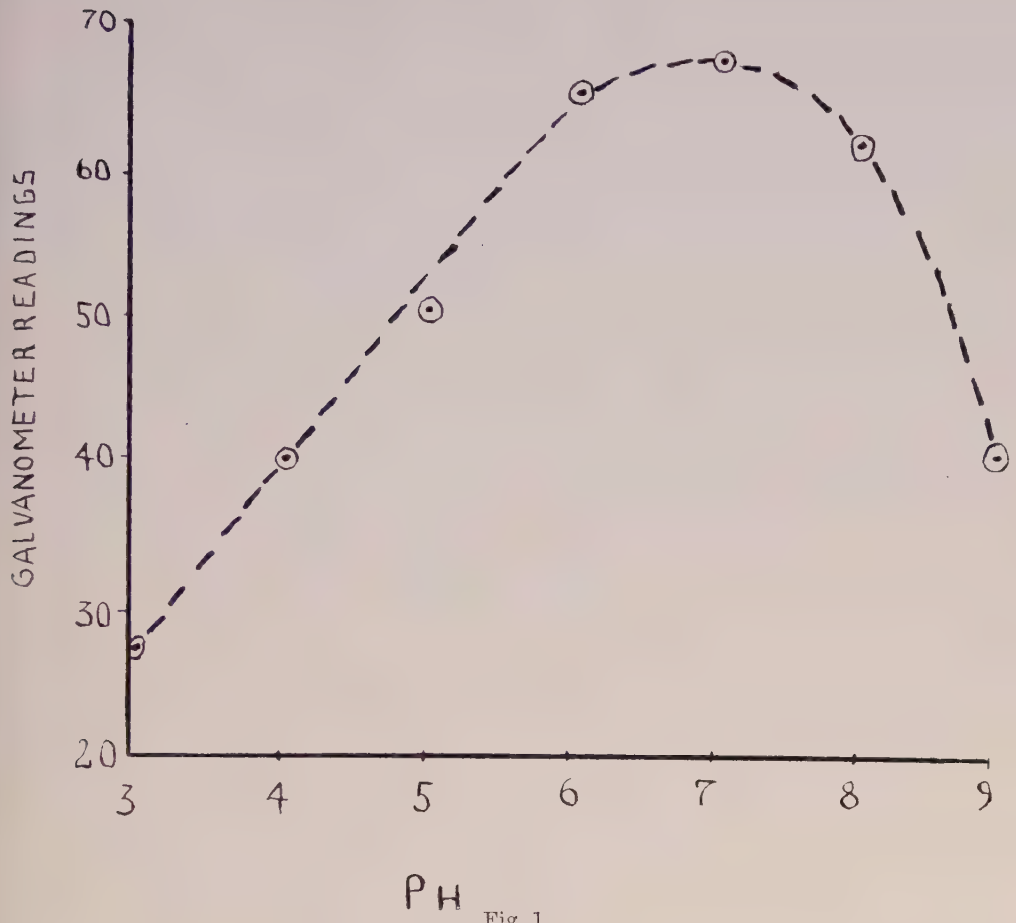

Fig. 1
Relationship of the enzyme activity to pH

II. *Measurement of activity of the bacterial suspension.* A series of tubes was prepared, each containing 5 ml of a standard solution of nicotinic acid (10 μg per ml), 0.5 ml of 0.2 M phosphate buffer of pH 6.0, and 0.05 ml of bacterial suspension. These were allowed to stand at room temperature for 10 to 30 min. and the enzyme was then inactivated by immersion in a water bath at 90°C for 5 min. The tubes were centrifuged for 15 min. at 10,000 r.p.m. and 5 ml of the clear supernatant fluid were analyzed for nicotinic acid by a colorimetric method§ employing cyanogen bromide and p-phenylenediamine. The intensity of the yellow color was measured in a Coleman spectrophotometer at a wave-length of 420 mμ.

§ The procedure used for the estimation of nicotinic acid will shortly be published elsewhere.

Experimental. I. *Activity of the culture.* It was found that 50 μg of nicotinic acid were destroyed in 15 min. at room temperature by 0.05 ml of the bacterial suspension. Under the same conditions there was no appreciable decomposition of nicotinamide.

II. *Effect of pH.* Aliquots of the substrate were adjusted to pH values ranging from 3 to 9. These were inoculated and the amount of nicotinic acid remaining was determined. The results shown in Fig. 1 indicate that the optimum pH was about 7.0.

III. *Attempt to obtain a cell-free enzyme.* Freezing and thawing a sample of the bacterial suspension 8 times in 24 hours destroyed 66% of the normal activity, as is shown in Table I. Experiments are now in progress to obtain cell-free enzymes by other methods.

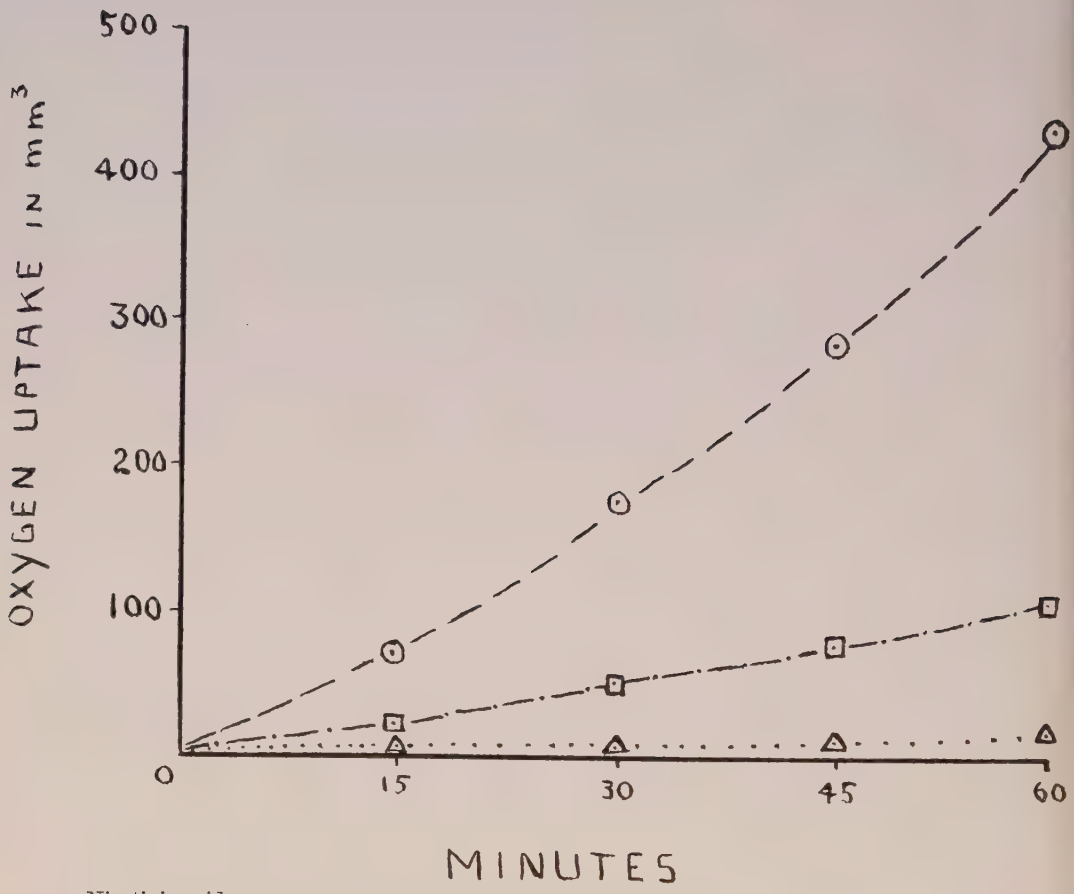

FIG. 2.
Oxygen uptake of *Ps. fluorescens* 30 with nicotinic acid as influenced by sodium azide and hydroxylamine hydrochloride (M/60). Temperature 27°.

TABLE I.
Effect of Alternative Freezing and Thawing on Enzyme Activity.

Time in min.	μg nicotinic acid destroyed by (1) normal cells	(2) treated cells
5	29.6	5.4
10	47.0	9.9
15	50	17.0
30	—	22.3

IV. *Mode of destruction of nicotinic acid.* Warburg experiments showed that the destruction of nicotinic acid was accompanied by oxygen consumption. There was a decrease in the oxygen consumption when the amount of the substrate was decreased. The experiments were conducted at 27°C with M/15 phosphate buffer of pH 7 as medium. The total volume of liquid in the vessels was 2.3 ml; 0.3 ml of bacterial suspension were employed. The center well contained 0.2 ml of 50% KOH.

A nicotinic acid solution containing 615 μg was tipped into the main vessel from one side arm after equilibrium had been reached. After 75 minutes the oxygen uptake was determined and the reaction was stopped by the addition of 0.5 ml of concentrated sulphuric acid from the second side arm. The residual nicotinic acid was determined colorimetrically. For the oxidation of 0.475 micromoles of nicotinic acid 1.42 micromoles were required, *i. e.* 3 moles of oxygen per mole of

nicotinic acid. In a similar experiment 0.0475 micromoles of nicotinic acid were destroyed and 0.13 micromoles of oxygen were taken up. This corresponds to 2.7 moles of oxygen per mole of nicotinic acid.

Sodium azide and hydroxylamine inhibited the oxidation drastically, as is seen in Fig. 2. The manometric measurements were verified with the colorimetric method. In another test, the detergent, lauryl pyridinium chloride‖ in a concentration of 0.0013%, brought about complete inhibition. Octyl alcohol and toluene inhibited the oxidation 68 and 80%, respectively, as indicated with the colorimetric method. There was no oxygen uptake with nicotinamide as substrate.

Summary and Conclusion. The destruction of nicotinic acid by *Pseudomonas fluorescens*, previously described by Koser and Baird[3] was found to be an enzymic oxidation. Utilization of nicotinic acid is accompanied by oxygen uptake at a ratio of about 3 oxygen to 1 nicotinic acid. Nicotinamide is not attacked by the oxidase under our experimental conditions. Two types of inhibitors interfere with the enzymatic destruction of nicotinic acid: inhibitors of heavy metal enzymes, such as sodium azide and hydroxylamine, and surface active agents, such as octyl alcohol, toluene and lauryl pyridinium chloride. The rate of oxygen consumption indicates a destruction of the nicotinic acid molecule. Since nicotinic acid serves as a substrate and not as a co-enzyme it should be noted that this reaction sets it apart from its known function in the co-factors.

‖ We wish to thank the Hooker Electrochemical Corporation, Niagara Falls, N.Y., for a sample of lauryl pyridinium chloride.

15986

Effect of Age in Guinea Pig on Local Passive Sensitization and Skin Reactions Toward Histamine.

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Young guinea pigs are universally accepted as being more suitable for the study of anaphylactic reactions than old animals. Thomsen,[1] who confirmed the work of others, found old guinea pigs more difficult to sensitize than young guinea pigs. He also studied the effect of age on the passive sensitivity developed after injection of anti-horse rabbit serum. Sensitivity was measured by injecting the antigen intravenously. His results showed that the young guinea pigs became more sensitive than the old guinea pigs.

To determine whether age had a similar effect on the production of local anaphylaxis, a comparison was made of the ability to transfer sensitivity passively to the skin of old and of young animals. The method recently described by Chase[2] for producing local passive sensitization in the skin of the guinea pig was used for making these comparisons.

Since a tissue liberation of histamine or histamine-like substance is thought to occur during anaphylactic reactions,[3,4] the response to intradermal injection of this substance in young and in old guinea pigs was studied.

Material. All of the animals were albinos and were obtained from a single breeding colony. Animals of both sexes were used. The old animals were 2 to 4 years and the young

1 Thomsen, Olof, *Z. f. Immunitats. forsch.*, 1917, **26**, 213.

2 Chase, M. W., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 238.

3 Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1910, **41**, 318.

4 Lewis, T., *Brit. M. J.*, 1926, **2**, 61.

TABLE I.
Local Passive Transfer of Anti-protein Serum to the Skin of Young and of Old Guinea Pigs.

Exp. No.	No. of animals	Mean wt	Age	Dose of anti-protein serum	Latent period before injecting antigen, hr	Mean dose of antigen inj. intraäbdom.	No. with positive reactions	% positive	Mean dimensions of reactions in mm	System
1	12	553	10 wks	0.1 cc undil. serum	24	126 mg	8	67	21.6x24.1x1.1	Azoovalbumin-anti-azoovalbumin
	12	960	2-4 yrs	"	24	189 "	0	0	0	"
2	5	487	8 wks	0.05 cc 1:4 dilution	24	1.0 cc	5	100	17.8x18.4x1.3	Horse serum-anti-horse serum
	5	1159	2-4 yrs	"	24	2.3 "	1	20	8.0x11.0 f*	"
	10	948	2-4 "	"	48	1.6 "	2	20	8.5x10.0 f	"
3	5	477	8 wks	"	96	1.0 "	2	40	11.0x12.0 f	"
	5	1158	2-4 yrs	"	96	2.3 "	1	20	8.0x8.0 f	"
4	5	398	6 wks	0.1 cc 1:2 dilution	24	0.6 "	4	80	23.8x26.0x1.5	"
	5	1066	2-4 yrs	"	24	1.7 "	1	20	18.0x22.0x0.5	"
5	5	317	5 wks	"	24	intradermally 0.1 cc undil. horse serum	5	100	normal site 21.0x22.8x2.0 sensitized site† 36.0x40.5x5.0	"
	10	1193	2-4 yrs	"	24	"	3	30	normal site 16.0x19.3x3.3 sensitized site 20.3x24.3x1.0	"

* Erythema only, no edema.
† Mean dimensions in animals in which reactions were elicited.

or young adult animals were 4 to 10 weeks of age.

Anti-azoovalbumin guinea pig and anti-horse guinea pig serum were the antiserums employed. The method described by Heidelberger, Kendall, and SooHoo[5] was followed for preparing the azoovalbumin. The anti-azoovalbumin was prepared by injecting 50 mg of the antigen intraabdominally into old guinea pigs. The animals were bled 44 days after the sensitizing dose of antigen was given. The anti-horse guinea pig serum was prepared by injecting 0.01 cc of horse serum intraabdominally into 600 g guinea pigs. These animals were bled 21 days after administration of the antigen.

The histamine dihydrochloride solutions were freshly prepared in saline just before use.

Experimental. The antiserums were injected into the closely clipped skin of the backs of both the young and the old animals. After varying intervals of time the corresponding antigens were injected. In experiments 1, 2, 3, and 4 (Table I) these antigens were administered intraabdominally. The dose of azoovalbumin administered to the young animals was 126 mg. The old animals received 189 mg. The dose of horse serum injected was 0.15 to 0.2 cc for each 100 g of body weight. The reactions were measured 15, 30, 45, 60, 90, and 120 minutes later. In most instances they were fully developed or reached their maximum intensity in 60 minutes; consequently, the mean diameters of these 60-minute values are the only measurements given. In agreement with Chase, reactions were not elicited in all of the young animals. The data given in Table I show that the skin of young guinea pigs is more easily sensitized than the skin of old guinea pigs.

Experiment 5 (Table I) was conducted differently than the first 4 experiments because we thought it conceivable that the absence or low sensitivity in old animals could be accounted for by a slow rate of absorption of horse serum. In this experiment, 0.1 cc of a 1:2 dilution of anti-horse guinea pig serum was injected on one side of the back. For a control 0.1 cc of a 1:2 dilution of normal guinea pig serum was injected into a corresponding site on the other side of the back. Twenty-four hours later, 0.1 cc of horse serum was injected intradermally into each site. The two reactions were read 15, 30, 45, and 60 minutes after administration of the antigen. The skin was considered to be sensitized in those animals in which edema occurred at the sites previously injected with the antiserum. In these animals, the skin reactions at the sites of the injections of the antiserum were significantly larger than the blebs formed by the injections of normal horse serum.

The results of these experiments show that there was no significant difference in the number of old animals that reacted whether the antigen was given intradermally at the site of injection or given intraabdominally.

Activity of histamine dihydrochloride injected into the skin of old and of young guinea pigs. Three groups of old and young closely clipped guinea pigs were injected intradermally with 0.1 cc of varying dilutions of histamine (Table II). The histamine was injected on one side and 0.1 cc of saline, for control, was injected at the corresponding site on the other side of the back. The pH of the 1:10,000 dilution of histamine was 4.8. The pH of the saline control for this dilution was brought to this value by the addition of Sorensen's sodium citrate-hydrochloric acid buffer pH 4.8 in the proportion of one part of buffer to 39 parts of the saline. The pH of the 1:100,000 and 1:1,000,000 dilutions of histamine was approximately that of the saline used for the control injection and consequently needed no adjustment. The reactions, localized edematous areas, were read 15, 30, 45, and 60 minutes after injection. All of the old and young animals injected with the 1:10,000 and 1:100,000 dilutions of histamine reacted. It was observed that the reactions, in most instances, reached their maximum intensity 30 minutes after injection. The 30-minute readings, being the most

[5] Heidelberger, M., Kendall, F. E., and SooHoo, C. M., *J. Exp. Med.*, 1933, **58**, 137.

TABLE II.
Cutaneous Response to Intradermal Injection of Various Concentrations of Histamine Dihydrochloride in Young and Old Guinea Pigs.

No. of animals	Mean wt	Age	Dose	Mean size of reactions in mm		Ratio of areas
				Histamine	Saline	
5	337	5 wk	1:10,000	22.4x24.4	14.0x14.4	2.7
5	1080	2-4 yr	"	16.6x19.6	10.2x13.4	2.4
10	375	6 wk	1:100,000	17.3x19.6	13.4x15.4	1.6
10	1080	2-4 yr	"	16.6x17.7	12.5x14.5	1.6
5	421	7 wk	1:1,000,000	13.8x15.8	13.6x15.4	1.0
5	1145	2-4 yr	"	14.6x15.2	13.8x15.0	1.1

satisfactory, were the only ones given consideration for purposes of comparison. The injections of saline into the skin of young animals and that of old animals does not produce the same size blebs. For this reason, in analyzing the data from the experiment, the size of the bleb formed by injection of the saline control must be considered in relation to the size of the corresponding histamine wheal. Comparison between the ratios of the areas of the saline and histamine wheals observed in old and in young animals is made, therefore, rather than a comparison of the areas themselves. On this basis of comparison there is no significant difference between the reactions elicited to histamine in the old and in the young guinea pigs.

Discussion. The foregoing experiments show that there is a marked difference in the ability of anti-protein serums to sensitize the skin of old and of young guinea pigs.

The reactions in the two age groups toward histamine were studied to determine whether a weaker response toward histamine could account for the difference in reactivity. The results show that both old and young guinea pigs react in the same degree. It is of interest to note that Lamson and Pope[6] and Darsie, *et al.*[7] were unable to produce reactions in the skin of the guinea pig with concentrations of histamine as high as 1:100. Ramsdell[8] on the other hand, demonstrated reactions with a 1:1,000,000 dilution of histamine.

We have no explanation for the inability of Lamson and Pope and of Darsie, *et al.* to obtain positive reactions to histamine other than that suggested by Follensby and Hooker[9] that some strains of guinea pigs may be more reactive toward histamine than others.

Summary. The skin of old guinea pigs is more difficult to sensitize passively than the skin of young guinea pigs. This difference in response is not due to differences in the reactivity of old and of young guinea pigs toward histamine since both old and young guinea pigs react toward this substance to the same degree.

[6] Lamson, R. W., and Pope, M. L., *J. Immunol.*, 1927, **14**, 365.

[7] Darsie, M. L., Perry, S. M., Rosenfeld, D., and Zaro, J. A., PROC. SOC. EXP. BIOL. ADN MED., 1945, **59**, 278.

[8] Ramsdell, Susan G., *J. Immunol.*, 1928, **15**, 305.

[9] Follansby, E. M., and Hooker, S. B., *J. Immunol.*, 1944, **49**, 353.

15987

Susceptibility of the English Sparrow (*Passer domesticus*) to Infection with Psittacosis Virus of Pigeon Origin.

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The demonstration of ubiquitous infection among feral pigeons with psittacosis or psittacosis-like viruses[1,2,3] emphasizes the possibility of infection in the common English or house sparrow *(Passer domesticus)* because of its habit of feeding in association with pigeons. Various other members of the order Passeriformes have been shown to be naturally infected with psittacosis virus.[4]

Attention also was directed toward this possibility by an illness diagnosed serologically in this laboratory as psittacosis which was encountered in a woman physician who had handled a sick English sparrow. The onset of illness was on December 2, 1945 just 2 weeks after the patient had killed a sick and emaciated English sparrow which had frequented a bird feeding tray at her home. The patient also had scraped and cleaned the tray of droppings. A few pigeons belonging to a neighboring loft were noticed nearby occasionally. The illness was not severe and consisted of fever lasting for 6 days, (the highest temperature was 102.5°F) chills, severe sweats, and a dry cough. Abnormal physical findings were limited to the presence of rales over the lung bases. A roentgenogram of the chest taken 15 days after onset revealed an area of increased density at the left base and an increase in width of the hilar shadow. The specific diagnosis was not considered during the acute illness so that material for virus isolation studies was not available. Serum taken 29 days after onset fixed complement in the presence of both psittacosis antigen and commercial *Lymphogranuloma venereum* antigen (Lygranum) in a dilution of 1:32. Serum taken 10 weeks after onset also fixed complement in the presence of psittacosis antigen but not in the presence of Q fever antigen. Samples of serum which had been collected for another purpose 3 and 5 months prior to the onset did not fix complement. While it was not possible to state definitely that the source of virus was the English sparrow, the question was considered worthy of further study.

Efforts to investigate the susceptibility of the English sparrow to psittacosis virus were undertaken in two ways. The first was to seek evidence of natural infection by attempts to recover the virus from birds captured in the vicinity of Washington, D. C. and by testing their serum for the presence of complement fixing antibodies. The second was to test directly the susceptibility of this species by the inoculation of captured birds with a known strain of virus.

Sparrows were captured in a wire funnel trap in 4 different locations in the Washington, D. C. area during 1946 and 1947. The spleen, liver, and a kidney of each bird to be tested for the presence of virus were ground together in a mortar, and a 10% suspension in 0.85%, NaCl solution prepared. This was inoculated intraperitoneally into stock white mice (NIH strain). A week or ten days later the mice were autopsied and a normal saline suspension of the spleen inoculated intracranially into a second lot of mice. A third transfer was routinely made intracranially.

Approximately half of the birds were first bled from the heart by passing a 22-gauge needle through the suprasternal notch in the midline and parallel to the vertebral column. Complement fixation tests of the serum were performed according to the technique devised

1 Zichis, J., Shaughnessy, H. J., and Lembke, C., *J. Bact.*, 1946, **51**, 616.

2 Labzoffsky, N. A., *Canad. J. Pub. Health*, 1947, **38**, 86.

3 Davis, D. J., and Ewing, C. L., *Pub. Health Rep.*, 1947, **62**, 1484.

4 Meyer, K. F., *Medicine*, 1942, **21**, 175.

TABLE I.
Results of Inoculation by Different Routes of the English Sparrow with Psittacosis Virus of Pigeon Origin.

No. of bird	Route of inoculation	Result in days after inoculation	Complement fixation titer of bird serum	Recovery of virus from bird organs by intracranial mouse inoculation		
				Brain	Spleen and liver combined	Kidney
965-1	intracranial	dead 3 days	—	+	0	0
965-2	"	" 3 "	—	+	+	0
965-3	"	" 9 "	—	+	+	+
965-4	"	" 15 "	—	+	+	—
990-1	intraperitoneal	" 7 "	—	—	+	+
990-2	"	" 15 "	—	+	+	+
1047-3	oral-gastric	sacrificed 14 days	1/64	—	+	0
1047-4	" "	" 14 "	0	—	0	0
1057-1	" "	dead 10 days	—	—	0	0
1057-2	" "	sacrificed 14 days	0	—	+	+
1057-3	" "	" 14 "	1/256	—	0	0
1106-2	" "	" 14 "	0	—	0	0
1118-5	" "	" 14 "	0	—	0	0
1118-6	" "	" 14 "	0	—	0	0
1119-2	" "	dead 9 days	—	—	0	0
1119-3	" "	" 11 "	—	—	+	+
1119-4	" "	" 11 "	—	—	+	+
1119-5	" "	sacrificed 11 days	0	—	+	+
1144-1	" "	dead 8 days	—	—	+	+
1144-2	" "	sacrificed 14 days	0	—	+	+
1144-3	" "	" 14 "	0	—	0	0

+ Virus recovered.
0 Virus not recovered.
— Not attempted.

by Bengtson[5] for rickettsial diseases. The antigen was prepared from psittacosis virus recovered from a parrot, grown in allantoic fluid of the developing chick embryo and killed with formalin or phenol.

Psittacosis or psittacosis-like virus was not isolated from any of the 103 sparrows examined. In two instances an unidentified bacillus apparently originating in the birds caused the death of the mice. No complement fixing psittacosis antibodies were detected in the 59 samples of serum tested.

Experimental Infection. In order to investigate the susceptibility of this species to infection with psittacosis virus sparrows were inoculated by 3 different routes with virus recently isolated from a feral pigeon. A total of 37 birds were inoculated, 4 intracranially under ether anaesthesia, 2 intraperitoneally, and 31 by dropping the inoculum into the throat or by passing a blunt needle down the esophagus of the anaesthetized bird. Due to trauma and the difficulty of keeping wild birds, 16 of the 31 inoculated by the latter method died within a few days and are not included in Table I.

The experimental birds were captured in the wild state, but the chance of natural infection was probably less than 1 in 100 since no virus had been found in 103 birds trapped at the same times and places. The virus inoculum was a saline suspension of mouse brain, yolk sac or allantoic fluid of the developing chick embryo infected with the same strain (No. 26) of pigeon virus.

White mice inoculated intracranially with each inoculum as a control died, and impression smears of the brain stained by Machiavello's stain showed typical clusters of elementary bodies in each instance.

Table I shows the results of inoculation of virus into the 21 birds which survived the trauma of capture and inoculation. Birds surviving until the 14th or 15th day after inoculation were bled and the serum tested for complement fixing antibodies. The sacrificed birds and those which died were autopsied, and tested for the presence of

[5] Bengtson, Ida A., *Pub. Health Rep.*, 1944, **59**, 402.

virus by inoculating mice intracranially with an approximate 10% suspension of the organs in 0.85%, NaCl solution. The presence of the characteristic clusters of elementary bodies in impression smears of the brain of mice dying 3 to 7 days after inoculation with the suspected material was taken as evidence of the presence of virus in the organ tested.

Birds inoculated intracranially died 3 to 15 days later and showed no gross abnormalities. Virus was present in the kidney and combined liver and spleen as well as in the brain.

In birds inoculated intraperitoneally the spleen was enlarged and soft, and the liver showed gross areas of focal necrosis, while the remaining organs appeared normal. Virus was recovered from the brain, kidney, and combined liver and spleen.

The oral-gastric route of infection was selected as approximating a natural mode of bird to bird transmission of virus. It was recovered from the organs of 7 of the 15 birds inoculated by this route which survived the first 7 days, and was present in the kidneys of 6 of these. Serum from 2 birds killed on the 14th day, from only one of which virus was recovered, fixed complement in the presence of psittacosis antigen, but serum from 2 others from which virus was recovered did not fix complement. Six other samples from uninfected birds were negative. The failure to demonstrate antibodies in birds from which virus is recovered may be due to a delay in antibody production as has been observed in pigeons.[6]

Summary. A serologically diagnosed case of psittacosis following exposure to a sick English sparrow and its droppings suggested an investigation of this species of bird as a carrier of psittacosis virus. 103 sparrows were examined for presence of active virus and serum from 59 of them were tested for complement fixing antibodies, but no evidence of natural infection was obtained. Four birds inoculated intracranially, 2 inoculated intraperitoneally and 7 of 15 inoculated by the oral-gastric route with psittacosis virus of pigeon origin became infected and virus was recovered from their organs.

6 Meyer, K. F., Eddie, B., and Yanamura, H. Y., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 609.

15988

Tyrosine Tolerance Test in Pregnancy and the Puerperium.*

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We have shown previously that in normal pregnancy there is a marked increase in the excretion of both ingested and intravenously administered histidine, and that this is associated with a reduction of renal tubular reabsorption as well as with a delayed gastrointestinal absorption of this particular amino acid.[1,2] There is no apparent change, however, in the rate at which histidine disappears from the blood stream. The strange rejection of this important amino acid by both the renal and gastrointestinal epithelium during normal pregnancy is unexplained; we wished, therefore, to compare this finding with the metabolism and excretion of another amino acid, tyrosine, by the maternal organism. It would be desirable to know, furthermore, whether a tyrosine tolerance test might be of use as a measure of liver function in the toxemias of late pregnancy.

Following the oral ingestion of as much as

* Investigation aided by a grant from the John and Mary R. Markle Foundation, New York.

1 Page, E. W., *West. J. Surg.*, 1943, **51**, 482.

2 Page, E. W., *Am. J. Obstet. and Gynec.*, 1946, **51**, 553.

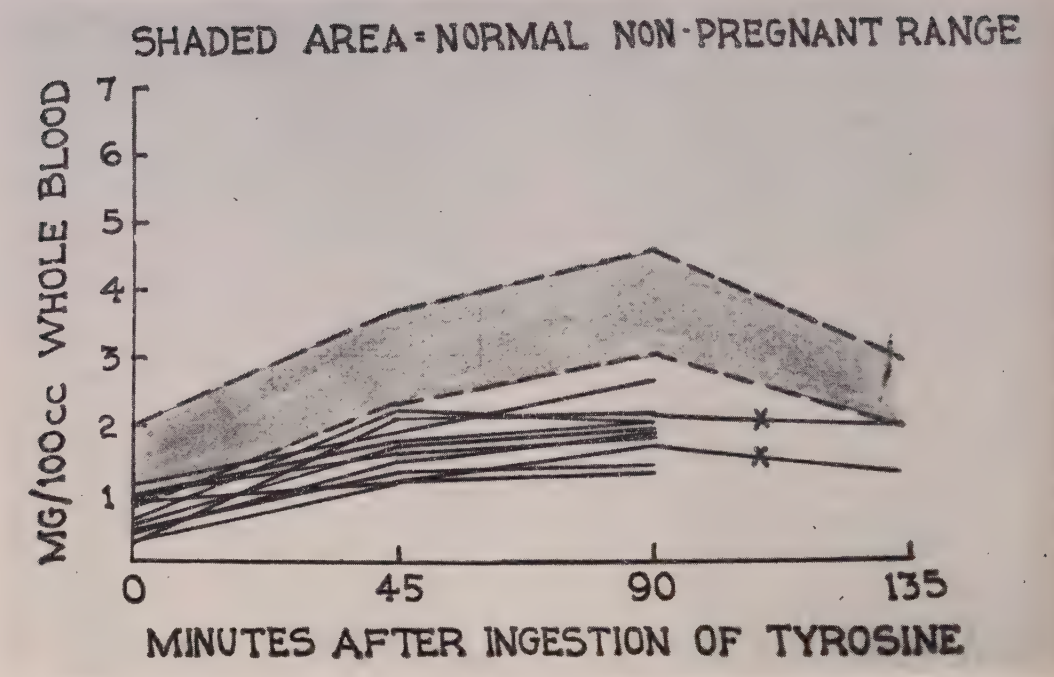

FIG. 1.
Tyrosine tolerance curves on 8 normal pregnant women and 2 women with severe toxemia (indicated by x). All values in 10 normal non-pregnant women fell within shaded area.

10 g of tyrosine by normal individuals, there are no significant changes in the urinary output of tyrosyl compounds.[3] The shape and height of the blood curve observed after such ingestion is therefore dependent upon two factors: the rate of absorption from the gastrointestinal tract and the rate of metabolism (utilization or destruction) of this compound. Bernhart and Schneider[4] followed the tyrosyl concentration of the blood at hourly intervals after the oral administration of 4 g of tyrosine, and suggested that this procedure be used as a test of liver function. The impaired metabolism of tyrosine associated with diseases of the liver results in a high initial level followed by an abnormal increase in the blood level at one hour and a delayed return to normal. In this respect, the type of curve obtained is analogous to the glucose tolerance curve associated with impaired carbohydrate metabolism. When there is delayed absorption from the gastrointestinal tract, on the other hand, as noted in patients with untreated pernicious anemia, the peak is simply delayed until about three hours after ingestion but is not abnormally elevated.[5]

Methods. Eight volunteer subjects with normal pregnancies of 7 to 9 months' duration, one patient with eclampsia and one with a severe grade of preeclampsia were selected for study. Seven additional women with normal pregnancies were studied from 1 to 36 hours after delivery. Ten control subjects of similar age were selected from preoperative patients on the gynecologic service. All of the latter group were in good general health with no history of liver disease. Each woman was fasting on the morning of the test, and was given 4 g of tyrosine in casein solution. The methods of analysis employed, based upon the Millon reaction, were identical with those of Bernhart and Schneider,[4] except that blood samples were taken at intervals of 45 minutes

[3] Medes, G., *Biochem. J.*, 1932, **26**, 917.

[4] Bernhart, F. W., and Schneider, R. W., *Am. J. Med. Sci.*, 1943, **205**, 636.

[5] Swenseid, M. E., and Bethell, F. H., *Proc. Centr. Soc. Clin. Res.*, 1944, **17**, 40.

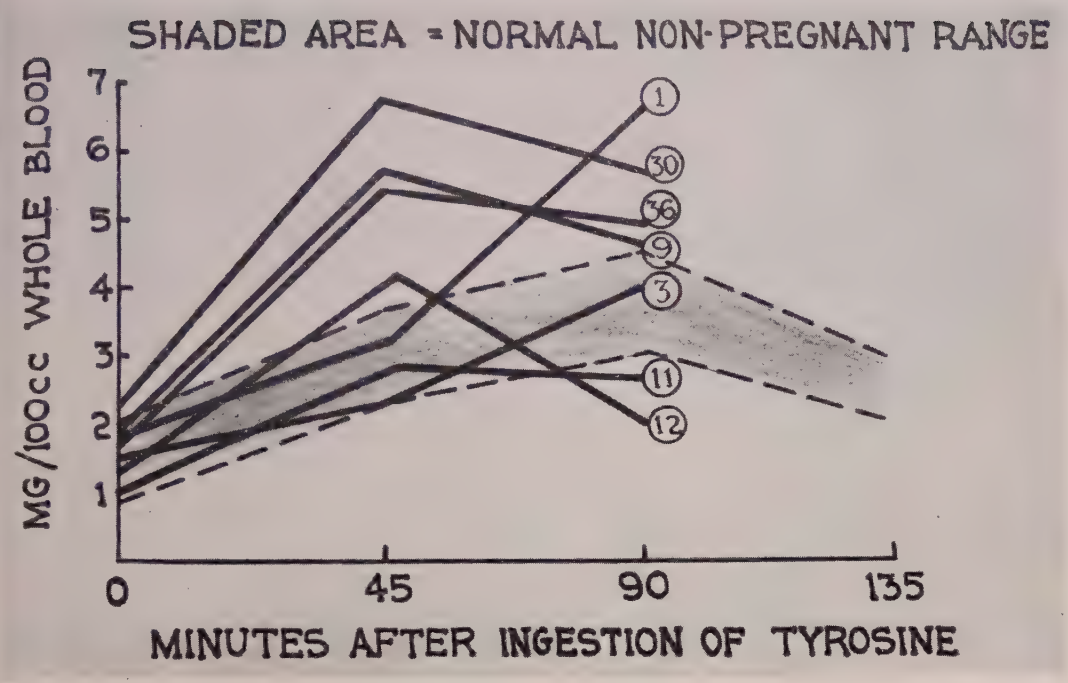

FIG. 2.
Tyrosine tolerance curves on 7 normal puerperal women. Figures in circle indicate the number of hours after delivery.

instead of hourly. This resulted in a change in the height and shape of the normal curve. In half the subjects of the normal non-pregnant and pregnant groups, urine samples were collected for a period of 8 hours before and 8 hours after administration of the tyrosine. These were analyzed for tyrosyl compounds by the Millon reaction as developed by Folin and Ciocalteu[6] and modified for urine determinations by Medes.[3]

Results. The fasting blood tyrosyl concentration of the 10 non-pregnant women was 1.44 mg/100 ml ± 0.09, with a standard deviation of 0.28. This compares favorably with the value of 1.4 as previously determined by the same method,[4] and with the value of 1.48 as determined by Hier and Bergheim[7] using microbiological methods. The fasting blood level of the 10 pregnant women was definitely lowered, being 0.99 mg/100 ml ± 0.12 with S.D. of 0.37. The difference between these means is 3 times the standard error of the difference and may be considered significant. On the other hand, the postpartum values were even higher than the non-pregnant level.

The tyrosine tolerance curves for the 8 normal pregnant women and the two toxemia cases are illustrated in Fig 1. It may be seen that the values remained low, but that the rate of rise is similar to that of normal non-pregnant women, though tending perhaps to be somewhat flatter. There was no apparent delay in gastro-intestinal absorption as described for cases of pernicious anemia. The average urinary output of tyrosyl compounds before the test was 147 mg/12 hrs, and this increased to an average of only 190 mg/12 hrs after the test. There were occasional falls rather than rises, and there were no discernible differences between the pregnant subjects, with or without toxemia, and the normal non-pregnant women.

Soon after delivery, however, there ap-

[6] Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, 1927, **73**, 627.

[7] Hier, S. W., and Bergheim, O., *J. Biol. Chem.*, 1946, **163**, 129.

peared to be a shift in the metabolism of tyrosine. The type of curves (Fig. 2) suggest impaired liver function, but it is more likely that the shift represents a reduced rate of utilization of this amino acid for protein synthesis in contrast to an increased utilization prior to delivery. This is in keeping with the well-known transition from a positive to a negative nitrogen balance after delivery. In the two subjects tested only 1 and 3 hours post-partum, the blood values were still rising at 90 minutes, suggesting a delayed absorption. This may represent a persistence of the known delay in both stomach emptying time and absorption of foodstuffs during active labor. The fact that pregnancy and the puerperium alter the tyrosine tolerance curves in opposite directions from the normal, and the failure to find further alterations in the 2 severe toxemia cases, would make it hazardous to use this procedure as a liver function test in pregnant or puerperal women.

Conclusions. 1. The fasting blood tyrosyl level is significantly lowered during normal pregnancy, but not in the puerperium.

2. The reduced tyrosine tolerance curve during pregnancy suggests an increased rate of metabolism for this amino acid. There is an immediate reversal of this effect after delivery.

3. Unlike histidine, the renal tubular reabsorption of tyrosine appears to be as complete in pregnant as in non-pregnant subjects.

4. No alterations in tyrosine tolerance were noted in a case of preeclampsia nor in a case of eclampsia.

5. The observed shifts in tyrosine metabolism, possibly concerned with similar shifts in rates of protein synthesis, would probably invalidate the tyrosine tolerance test as a measure of liver function in pregnant and puerperal women.

15989

Further Studies on Renal Hyperlipemia.*

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Previous work[1,2,3] has shown that in dogs and rats unilateral and bilateral nephrectomy and the parenteral administration of nephrotoxic agents like mercury bichloride, uranium nitrate and potassium dichromate are followed by an increased blood lipid concentration. This effect of bilateral renal ablation has been confirmed by Winkler and associates[4] in dogs, has been recorded by these authors in monkeys, and by Nekludow[5] in cats. Hyperlipemia has also been found in rats made experimentally nephritic either by injection of antikidney serum[6] or by immunization with streptococcus—kidney antigen.[7] Hence, the kidneys of dogs, cats, rats and monkeys seem to function as part of a mechanism which influences blood lipid concentration.

Whether hyperlipemia also follows nephrectomy or renal injury in human beings has been investigated, and animal experiments have been carried out in an attempt

* Aided by a grant from the John and Mary R. Markle Foundation.

1 Heymann, W., *Science*, 1942, **96**, 163.

2 Heymann, W., and Clark, E. C., *Am. J. Dis. Child.*, 1945, **70**, 74.

3 Heymann, W., and Sekerak, B., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 276.

4 Winkler, A. W., Durlacher, S. H., Hoff, H. E., and Mann, E. B., *J. Exp. Med.*, 1943, **77**, 473.

5 Nekludow, W. N., *Z. f. d. ges. exp. Med.*, 1925, **47**, 70.

6 Farr, L. E., Smadel, J. E., and Holden, R. F., Jr., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 178

7 Cavelti, P. A., and Cavelti, E. S., *Arch. Path.*, 1945, **40**, 163.

TABLE I.
Effect of Unilateral Nephrectomy on Serum Lipids in Man.

	Cholesterol		Total lipids	
	Lowest value Mg %	Highest value	Lowest value Mg %	Highest value
Before Nephrectomy	151	190	400	540
After "	129	200	310	560
Before "	114	182	490	650
After "	114	191	490	800
Before "	191	238	800	930
After "	102	237	700	930

TABLE II.
Effect of Oral Administration of Mercury Bichloride on Serum Lipids in Man.

Dose in g	Cholesterol, mg %		Total lipids, mg %		Lecithin, mg %	
	Lowest value	Highest value	Lowest value	Highest value	Lowest value	Highest value
1.0	210	258				
1.0	85	160	440	720		
2.0	52	100	290	400	136	223
3.0	100	116	370	490	167	186
2.5	35	150	470	620	173	221
2.5	180	272				

to clarify the mechanism of the hyperlipemia.

Methods. Cholesterol analyses were made according to Schoenheimer and Sperry's method[8] which had been adapted to use with a photoelectric colorimeter.[9] The total lipids were analyzed according to the gravimetric method of Wilson and Hammer;[10] phospholipids were determined after extraction according to the technic of Bloor, with the method of Benedict and Theis[11] and were calculated as lecithin.

Effect of Unilateral Nephrectomy on Serum lipids in Man. Three patients with hydronephrosis, pyohydronephrosis and renal tuberculosis, respectively, were subjected to unilateral nephrectomy. During a period of 4 to 8 days before operation, determinations of total lipids and total and free serum cholesterol were carried out 2 to 4 times and daily for 2 to 3 weeks after the removal of one kidney. (Table I) No postoperative hyperlipemia was observed.

Effect of Mercury Bichloride Poisoning on Serum Lipids in Man. Total lipid, cholesterol and phospholipid determinations were carried out each day or every other day for 5 to 14 days on the sera of 6 patients who had taken mercury bichloride orally. Four of the 6 suffered severe kidney damage and died of uremia. Table II shows that all of the lipid values were normal or low. The observations thus failed to confirm the results of Wichert, Jakowlewa and Pospeloff[12] who found high cholesterol values in the blood of 2 patients poisoned with mercury bichloride.

Effect of Repeated Intravenous Infusions on the Hyperlipemia observed in Dogs After Unilateral Nephrectomy. Because the 3 nephrectomized patients had received, after their operations, an intravenous infusion of 1000 cc of Ringer's solution and 1000 cc of 5% glucose solution, we investigated the effect in dogs of such infusions upon the development of the post-nephrectomy hyperlipemia. Three dogs were unilaterally nephrectomized†

8 Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, 1934, **106**, 745.

9 Fitz, F., *J. Biol. Chem.*, 1935, **109**, 523.

10 Wilson, W. R., and Hanner, J. P., *J. Biol. Chem.*, 1934, **106**, 323.

11 Benedict, S. R., and Theis, R. C., *J. Biol. Chem.*, 1924, **61**, 63.

12 Wichert, M., Jakowlewa, A., and Pospeloff, S., *Z. f. klin. Med.*, 1924, **101**, 173.

†We are greatly indebted to Dr. Harry Goldblatt of the Institute of Pathology, Western Reserve University, School of Medicine, for performing the operations on the dogs.

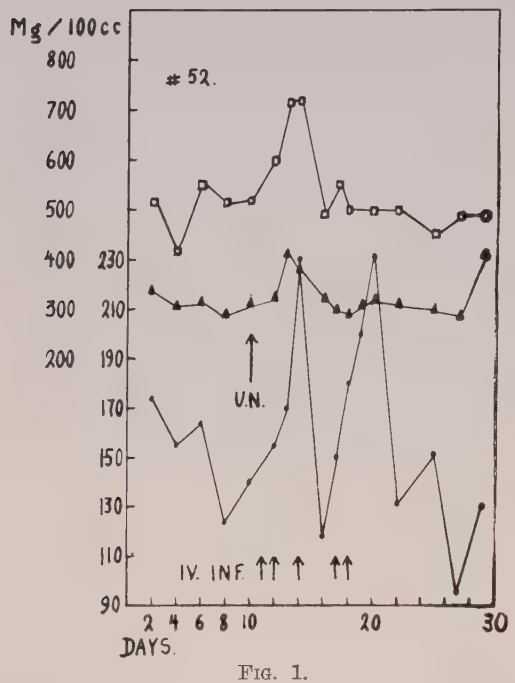

FIG. 1.

Effect of intravenous infusions of equal parts of Ringer's and 5% glucose solutions on serum lipids after unilateral nephrectomy in one of 3 dogs.

U. N., Unilateral nephrectomy.
Iv. Inf., Intravenous infusions.
Total Lipids, □——□
Lecithin, ▲——▲
Cholesterol, ●——●

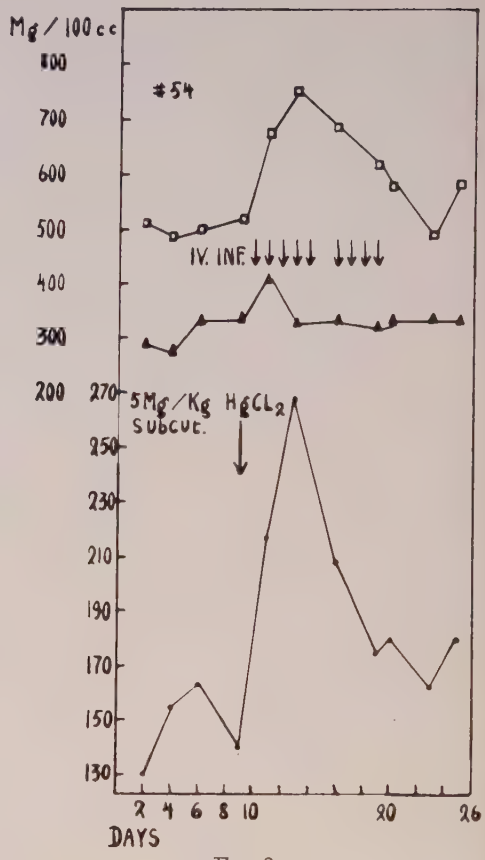

FIG. 2.

Effect of intravenous infusions of equal parts of Ringer's and 5% glucose solutions on hyperlipemia following injection of mercury bichloride in one of 3 dogs.

Iv. Inf., Intravenous infusions.
Total Lipids, □——□
Lecithin, ▲——▲
Cholesterol, ●——●

and given intravenous infusions every day or two after operation. Per kg body weight, 20 to 50 cc of equal parts of Ringer's and 5% glucose solution, were infused as in the nephrectomized patients. The infusion was by continuous intravenous drip for 1½ to 2 hours between 9 and 11 a.m. Blood samples were obtained for analysis before the infusions were started. Comparison of the results (Fig. 1) with those previously obtained[1,2] showed that the intravenous administration of the Ringer's and the glucose did not abolish, but did limit the extent of the post nephrectomy hyperlipemia. The changes in cholesterol, lecithin, and total lipid values observed were irregular and less marked in the dogs that received fluids after operation.

Effect of Repeated Intravenous Infusions on Hyperlipemia Observed in Dogs after Parenteral Administration of Mercury Bichloride. The effect of daily infusions on blood lipids was also studied in 3 dogs injected subcutaneously with 5 mg per kg body weight of mercury bichloride one day previous to the first infusion. Fig II shows that the infusions had little influence on the hyperlipemia development.

Effect of Repeated Oral Administration of Carbon Tetrachloride on Hyperlipemia Observed in Dogs after Subcutaneous Injection of Mercury Bichloride. In dogs and rats the oral administration of mercury bichloride had been previously shown[1,2] not to induce the hyperlipemia regularly observed after its parenteral administration. In both instances renal injury is produced. Inasmuch as mercury

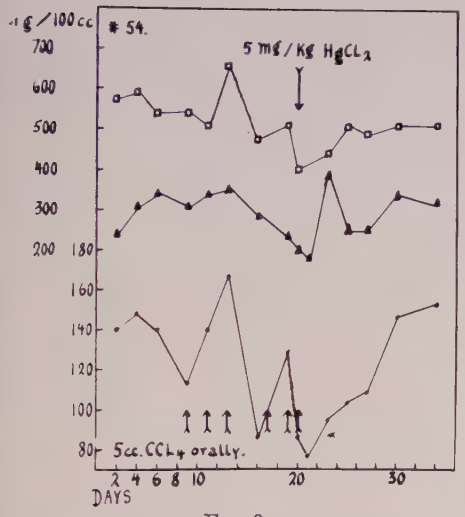

FIG. 3.

Effect on serum lipids in one of 3 dogs given carbon tetrachloride by stomach tube previous to a subcutaneous injection of mercury bichloride.

Total Lipids, □——□
Lecithin, ▲——▲
Cholesterol, ●——●

bichloride absorbed into the portal circulation might influence liver function, the effect of oral administration of carbon tetrachloride on the development of hyperlipemia was studied in dogs injected with mercury bichloride. After 5 cc of carbon tetrachloride had been given to 3 dogs by stomach tube at 2 to 4 day intervals for 12 to 18 days, 5 mg of mercury bichloride were injected subcutaneously. No elevation in blood lipid values was found in 2 of the dogs (Fig. 3). In the third, only a slight, delayed and irregular increase in total lipids and cholesterol was observed, and the phospholipids remained unchanged. The administration of carbon tetrachloride had thus inhibited the development of hyperlipemia otherwise observed after parenteral administration of mercury bichloride.

Discussion. The hyperlipemia observed in dogs after unilateral nephrectomy was not found in 3 human beings in whom one diseased kidney had to be removed. Although the conditions varied in the two series, it seems unlikely that the disparity is attributable to intravenous administration of fluids to the 3 nephrectomized patients after the operation. A possible explanation is that in the dogs, healthy kidneys were removed, whereas the kidneys removed from the 3 patients were severely diseased. In 2 of the 3 patients, two-thirds of the removed kidneys seemed to be intact, but only 2 to 3mm of cortex was left in the 3rd kidney. Unimpaired anatomical structure of about two-thirds of renal tissue, however, does not imply functional integrity. Welsh, Mellin and Taylor[13] have shown that certain functional changes accompanying the renal hypertrophy which follows unilateral nephrectomy in human beings, are not observed when the removed kidney is severely diseased.

In dogs and rats the parenteral administration of mercury bichloride is regularly followed by hyperlipemia. This is not the case, however, when the mercury bichloride is given by stomach tube. The results of oral administration in human beings are in agreement with those obtained in animals given mercury bichloride by stomach tube. Why hyperlipemia develops only after parenteral and not after enteral administration of mercury bichloride, despite the fact that renal injury is produced in both instances, remains to be clarified. Suggestive evidence was obtained that liver function could interfere with the development of hyperlipemia. Liver injury, produced experimentally by carbon tetrachloride in 3 dogs, inhibited the development of the hyperlipemia otherwise regularly observed after the parenteral administration of mercury bichloride.

Summary. 1. Determinations of total lipids, total and free cholesterol were carried out in 3 patients subjected to unilateral nephrectomy. No hyperlipemia was observed.

2. Total lipids, cholesterol and phospholipids were determined in the serum of 6 patients with mercury bichloride poisoning. The values were normal or low.

3. Daily intravenous infusions of equal parts of 5% glucose and saline solution in dogs after unilateral nephrectomy and subcutaneous injection of mercury bichloride had some influence on the post nephrectomy hyperlipemia.

[13] Walsh, G. A., Mellin, L., and Taylor, H. C., Jr., *J. Clin. Invest.*, 1944, **23**, 750.

4. Repeated oral administration of carbon tetrachloride prior to the subcutaneous injection of mercury bichloride in 3 dogs prevented the hyperlipemia in 2 of the animals.

15990

Hyperglycemia Induced by Certain Insulin Preparations.*

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In an attempt to demonstrate electroencephalographic changes characteristic of hypoglycemia, a preparation of insulin was injected intravenously into a paralyzed, artificially respirated cat at 30-minute intervals, the initial dose being one unit per kg body weight and each subsequent dose twice that of the preceding one. Four hours after the initial dose, no change in the electroencephalogram was evident. In similar experiments a transitory increase in blood glucose concentration usually followed each injection of insulin and hypoglycemia did not develop.

Since the cats used in the experiments considered above were paralyzed with dihydro-β-erythroidine hydrobromide and the blood glucose levels were high, the effect of intravenous injection of insulin preparations on the concentration of blood glucose was tested in anesthetized (amytal) and intact cats. Representative data are given in Tables I and II. In addition to the experiments summarized in the tables, others were carried out in which different times of collection of blood were used, no injection was made, other preparations of amorphous insulin were used, arterial blood was assayed, doses of insulin intermediate to and greater than those indicated were employed, and in which insulin was given subcutaneously and intramuscularly. The effect of intravenous injection of insulin during hypoglycemia, induced by insulin, and hyperglycemia, induced by administration of glucose, was also tested.

The results of the experiments may be summarized as follows. Intravenous injection of water or salt solution in volumes comparable to the volumes of insulin used provoked no marked change, or certainly no consistent change, in blood glucose level. With intravenous injection of all preparations of insulin tested (except NOVO) the blood glucose concentration increased, the maximum occurring 5-10 minutes after injection. The glucose concentration returned to the control level 20-30 minutes after injection at which time hypoglycemia began to develop. The increase in glucose concentration was evident with 0.1 unit of insulin per kg body weight and marked with one unit. The hyperglycemic effect seemed to be somewhat more pronounced in the anesthetized than in the intact or paralyzed cat.

The effect was apparently independent of the blood glucose level, *e.g.* intravenous injection of 2 units of a preparation of crystalline zinc insulin per kg when, after intramuscular administration of insulin, the blood glucose level was gradually falling and had reached 18 mg per 100 ml, was followed by an increase in glucose level to 82 mg per 100 ml and disappearance of electroencephalographic changes characteristic of hypoglycemia; likewise, intravenous injection of insulin, at high blood glucose levels, induced by administration of glucose, was followed by increases in glucose level. With subcutaneous and intramuscular administration of the several preparations of insulin no increase in blood glucose level occurred.

* Aided by grants from the Rockefeller and Josiah Macy, Jr., Foundations.

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TABLE I.

Effect of Intravenous Injection of Insulin on Blood Glucose Concentration of Intact Cats.

The cats were fasted about 24 hr. Just after collection of a control blood sample from a femoral vein, the material indicated was injected. Additional blood samples were obtained 5, 10, 20, and 40 minutes after the injection. Glucose was estimated by the method of Nelson.[2]

Substance injected	Dose, ml or units per kg bodywt	Blood glucose, mg per 100 ml				
		Control	5 min.	10 min.	20 min.	40 min.
Water	0.1	84	86	78	80	85
	0.3	79	82	84	78	79
	1.0	91	97	94	94	92
0.156 *M* NaCl	0.1	83	82	87	82	87
	0.2	85	84	86	79	80
	1.0	90	92	87	91	90
Crystalline zinc insulin, Lilly	0.1	67	70	70	41	24
	2.0	54	72	86	51	30
" " " Sharp & Dohme	0.1	70	81	76	64	55
	2.0	65	80	57	42	31
" " " Squibb	0.1	72	82	78	59	42
	2.0	61	83	81	56	38
Amorphous insulin, Squibb	0.1	71	89	86	54	43
	2.0	67	93	83	42	32
Crystalline zinc insulin,* Lilly, pH 7	0.1	65	70	71	63	52
pH 7	2.0	81	94	89	76	47
pH 11	2.0	103	135	119	106	75
pH 3	2.0	71	112	101	83	62
NOVO insulin	0.1	75	70	64	57	40
	1.0	78	70	61	52	24
	2.0	80	78	64	48	28

* Solid material dissolved in 0.01 *M* HCl followed by sufficient 0.01 *M* NaOH to give pH indicated.

Transitory hyperglycemia was a common occurrence following administration of earlier preparations of amorphous insulin, but with the advent of crystalline insulin it appeared that the hyperglycemic effect was due to impurities in the amorphous material.[3,4] Dr. R. Levine, with whom the findings considered above were discussed, pointed out that Duve[5] recently stated that intravenous injection of one insulin preparation (Lilly) had a hyperglycemic effect and that another (NOVO) did not. Thus, test of the latter preparation was made in anesthetized and intact cats. As indicated by the data in the tables and other experiments, injection of NOVO insulin in doses up to 10 units per kg body weight was followed only by a decrease in glucose level.

Test for hyperglycemic response in species other than the cat was not made in the present work. However, it has been stated that in the dog constant intravenous injection of certain insulin preparations has a hyperglycemic effect,[6] and that in man the fall of blood glucose level begins more rapidly following intravenous injection of NOVO insulin than with other preparations.[7] Further, the glycogenolytic effect of some insulin

[1] Olsen, N. S., and Klein, J. R., *Fed. Proc.*, 1947, **6**, 282.

[2] Nelson, N., *J. Biol. Chem.*, 1944, **153**, 375.

[3] Geiling, E. M. K., and DeLawder, A. M., *J. Pharm. Exp. Therap.*, 1930, **39**, 369.

[4] Burger, M., and Kramer, H., *Arch. exp. Path. Pharmakol.*, 1930, **156**, 1.

[5] Duve, C. de, *Glucose, Insuline et Diabète*, pp. XXVII, 307, Masson et Cie, Paris, 1945.

[6] Levine, R., and Caren, R., *Am. J. Physiol.*, in press.

[7] McCulloch, W. S., unpublished data obtained in this laboratory.

TABLE II.

Effect of Intravenous Injection of Insulin on Blood Glucose Concentration of Anesthetized Cats. The cats were anesthetized by subcutaneous injection of 0.07 g sodium amytal per kg body weight. The other experimental details were the same as indicated in Table I.

Substance inj.	Dose, ml or units per kg body wt	Blood glucose, mg per 100 ml				
		Control	5 min.	10 min.	20 min.	40 min.
Water	0.1	53	51	56	57	66
	0.3	95	85	86	96	93
	1.0	89	95	95	80	83
0.156 *M* NaCl	0.1	58	62	55	58	45
	0.2	61	63	50	77	56
	1.0	60	58	62	59	60
Crystalline zinc insulin, Lilly	0.1	74	90	81	62	50
	2.0	82	138	137	86	48
" " " Sharp & Dohme	0.1	61	78	62	50	37
	2.0	73	121	130	102	87
" " " Squibb	0.1	63	91	86	51	39
	2.0	72	115	122	115	72
Amorphous insulin, Squibb	0.1	59	73	56	40	24
	0.2	63	101	71	59	42
Crystalline zinc insulin, Lilly, pH 7	0.1	71	84	79	66	52
pH 7	2.0	93	120	109	97	63
pH 11	2.0	78	104	84	88	52
pH 3	2.0	66	98	110	95	66
NOVO insulin	0.1	66	64	66	54	23
	1.0	70	65	48	35	19
	2.0	68	66	69	56	43

preparations on rat and rabbit liver *in vitro* is not obtained with NOVO insulin.[8]

The cause of the increase in blood glucose following intravenous injection of certain preparations of insulin is not known at present and for many purposes, *e.g.* the therapeutic, it is of no importance. However, in experiments concerned with the effect of these preparations of insulin on metabolism *in vitro* or in carbohydrate balance studies following intravenous injection of insulin, the hyperglycemic effect should obviously be considered.

[8] Sutherland, E. W., and Cori, C. F., *Fed. Proc.*, 1947, **6**, 297. The observation cited was given in the amplified, verbal report.

Summary. Intravenous injection into cats of certain preparations of amorphous and crystalline insulin was found to provoke a transitory increase in blood glucose level. With one preparation, NOVO, no increase in glucose level occurred.

We are indebted to Dr. D. F. Robertson, Merck and Co., Inc., for a supply of dihydro-β-erythroidine hydrobromide; to Dr. L. Earle Arnow, Sharp and Dohme, Inc., for crystalline zinc and amorphous insulin; to Dr. K. K. Chen, Eli Lilly and Co., for solid crystalline insulin; to Terapeutisk Laboratorium A/S, Copenhagen, Denmark, for NOVO insulin; and to Eli Lilly and Co., for sodium amytal.

15991

Physical-Chemical Factors in Agglutination of Sheep Erythrocytes by Influenza Virus.*

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The literature contains conflicting data concerning the agglutination of sheep erythrocytes by influenza virus. McLelland and Hare[1] failed to obtain agglutination with either of the A or B strains used in their tests; Burnet[2] and Clark and Nagler[3] obtained agglutination with some influenza virus strains but not with others.

The data included in the present paper show that agglutination of sheep erythrocytes by the WS strain[4] of influenza virus is dependent upon physical-chemical factors; when the requirements are satisfied, sheep cells yield essentially the same results as chicken cells in tests for the presence of influenza virus, and for the demonstration of influenza virus antibodies by the agglutination-inhibition test. The WS strain seemed particularly well suited to studies concerning the effects of physical-chemical factors on agglutination of sheep erythrocytes by influenza virus because this strain has been found by others not to cause agglutination of sheep cells under the usual test conditions.

Materials and Methods. The stock virus suspensions consisted of pooled allantoic fluid obtained from 30 to 40 eggs containing 13- to 14-days-old embryos, and which had been inoculated beneath the chorio-allantoic membrane 2 days previously, with an egg adapted strain. The normal allantoic fluid was pooled from eggs containing embryos of the same age as those from which the infected fluid was obtained. The erythrocyte suspensions were prepared in saline after thorough washing, from citrated sheep blood which contained 0.04% formalin for bacteriostatic purposes. McIlvaine's phosphate-citric acid buffer[5] was used in all tests; it seemed to be superior to phosphate buffers for the present purposes.

All tests for hemagglutination were made after the method described by Hirst,[6] in 10 x 75mm precipitin tubes. The total volume of ingredients was 0.6 ml. The degrees of agglutination were read on the basis of the cell patterns on the bottoms of the tubes, after the tests had been at room temperature sufficiently long (about 2 hours) for the erythrocytes to completely settle; they are recorded as no agglutination (0), partial agglutination (+), and complete agglutination (++).

Results. Table I shows the results of an experiment made to determine the effect of pH upon agglutination of sheep erythrocytes by different concentrations of WS-infected, and normal allantoic fluids. Test mixtures consisted of equal volumes (0.2ml) of allantoic fluid (infected or normal), buffer, and ¼% suspension of sheep erythrocytes.

In tests (Table I) with 1-100 dilution of infected allantoic fluid the virus caused agglutination of sheep erythrocytes over most of the pH range tested. However, in tests with other concentrations of infected allantoic fluid, agglutination depended upon pH, and to some extent upon the concentration of al-

* This investigation was aided by a grant from the John and Mary R. Markle Foundation.

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2 Burnet, F. M., *Aust. J. Biol. and Med. Sc.,* 1942, **20**, 81.

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4 Smith, W., Andrewes, C. H., and Laidlaw, P. P., *Lancet,* 1933, **2**, 66.

5 Clark, W. M., *The Determination of Hydrogen Ions,* Baltimore, Williams and Wilkins Co., 1928, p. 214.

6 Hirst, G. K., *Science,* 1941, **94**, 22.

TABLE I.

Influence of pH upon Agglutination of Sheep Erythrocytes by Different Concentrations of Infected and of Uninfected Allantoic Fluids.

Initial dilution of allantoic fluid		pH of test mixtures																	
	Saline	8.0	7.8	7.6	7.4	7.2	7.0	6.8	6.6	6.4	6.2	6.0	5.8	5.6	5.4	5.2	5.0	4.8	4.6
Undil.	A	+	++	+	+	0	0	+	+	+	+	+	++	++	++	++	++	++	++
	B		++	0	0	0	0	0	0	0	0	0	0	0	0	0	P	P	H
1-5	A	0	0	0	0	0	0	0	+	0	+	+	+	+	+	+	+	+	0
	B		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	H
1-25	A	0	0	0	0	0	+	0	+	+	+	+	+	+	+	+	+	+	+
	B		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+
1-50	A	0	+	0	0	0	0	+	+	+	+	+	+	+	+	+	+	0	+
	B		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+
1-100	A	0	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B		0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	+	+
1-200	A	0	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B		0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	+	+
1-400	A	0	0	0	+	0	0	0	0	+	+	+	+	+	0	0	0	+	+
	B		0	0	+	+	0	0	0	0	0	0	0	0	0	0	0	+	+
No virus			0	0	+	+	0	0	0	0	0	0	0	0	0	0	0	+	+
No allantoic fluid																			

0 = No agglutination; + = partial aggl.; ++ = complete aggl; A = WS infected allantoic fluid; B = uninfected allantoic fluid; P = partial hemolysis; H = complete hemolysis.

lantoic fluid. Agglutination was caused by all dilutions of infected fluid, between pH 5.6 and pH 6.4; but in the more alkaline pH range the agglutination of sheep cells by virus was inhibited by substances present in the allantoic fluid, the degree of inhibition decreasing with decrease in concentration of allantoic fluid.

Buffer, in the absence of normal and infected allantoic fluids caused agglutination of the sheep cells between pH 4.6 and 5.0 and between pH 7.4 and 7.6; also, some agglutination was caused by concentrated normal and infected allantoic fluid at pH 7.8-8.0.

The combined effects of agglutination by virus, allantoic fluid and buffer (pH) on the one hand, and inhibition by allantoic fluid on the other, would render sheep cell tests unreliable over a large portion of the pH range tested. Nevertheless, at the pH range 5.8-6.0, sheep erythrocytes were so readily agglutinated by the virus that it seems likely that in a system adjusted to that pH, sheep erythrocytes should be satisfactory for influenza virus tests. It is to be noted, however, that concentration of cells is a very important factor. The best results are obtained with suspensions containing between ¼% and ½% of packed cells; very little agglutination is obtained with suspensions of 2% or more.

Table II compares sheep with chicken erythrocytes in tests to determine the virus content of allantoic fluid. The test mixtures contained equal volumes (0.2 ml), in the one instance of allantoic fluid (infected or uninfected), saline and ½% chicken cell suspension; and in the other instance, allantoic fluid, buffer pH 5.8, and ¼% sheep cell suspension.

It is clear (Table II) that the hemagglutinating effect of the infected allantoic fluid was approximately the same in tests with sheep cells as in tests with chicken cells.

Table III summarizes the results of an agglutination-inhibition test of known influenza virus antiserums versus the WS strain of virus. The pooled serums were from ferrets which had been repeatedly inoculated intranasally, in the one instance with strains of the virus of influenza A, and in the other instance

TABLE II.
Comparison of Chicken with Sheep Erythrocytes for the Determination of Virus Content of Allantoic Fluid.

Erythrocyte suspension		2-fold dilution of fluid 1	2	3	4	5	6	7	8	9	10
Chicken	A	++	++	++	++	++	++	++	+	0	0
	B	0	0	0	0	0	0	0	0	0	0
Sheep	A	++	++	++	++	++	++	++	+	0	0
	B	0	0	0	0	0	0	0	0	0	0

A = WS infected allantoic fluid; B = Uninfected allantoic fluid.
The systems in the case of sheep cell tests contained pH 5.8 buffer, those in the chicken cell tests contained saline.

TABLE III.
Comparison of Chicken with Sheep Erythrocytes for the Determination of Influenza Virus Antibodies by the Agglutination-inhibition Test.

Erythrocyte suspension	Ferret serum	Initial 2-fold dilution of serum 3	4	5	6	7	8	9	10	11	12
Chicken	infl. A	0	0	0	0	0	0	0	++	++	++
	infl. B	0	+	++	++	++	++	++	++	++	++
Sheep	infl. A	++	+	0	0	0	0	+	++	++	++
	infl. B	++	++	++	++	++	++	++	++	++	++

Titration of Virus Suspension Used in Test.

Erythrocyte suspension	Initial 2-fold dilution 1	2	3	4	5	6	7
Chicken	++	++	++	+	0	0	0
Sheep	++	++	++	++	0	0	0

The systems in the case of the sheep cell tests contained pH 5.8 buffer, those in the chicken cell tests contained saline.

with strains of influenza B. The test mixtures in the case of the sheep cell tests contained equal volumes (0.2 ml) serum dilution, WS allantoic fluid diluted 1:32 with pH 5.8 buffer, and ¼% sheep erythrocytes. The tests with chicken cells differed in that the WS allantoic fluid was diluted 1:32 with saline, and the chicken cell suspension was ½%.

It is clear from the data (Table III) that the inhibitory effect of influenza A antiserums on agglutination of erythrocytes by the WS strain was detectable with the sheep erythrocytes as readily as with chicken cells. The inhibitory effect of the serum in the dilutions 2^{-4} to 2^{-9} in both instances must have been specific, because it was not obtained with influenza B virus antiserums. It is interesting that the non-specific effect of the serums (dilutions 2^{-3} and 2^{-4}) differed in the 2 tests. In the case of tests with chicken cells, the serum caused inhibition of the virus hemagglutination. In the sheep cell tests, however, the serum itself agglutinated the erythrocytes.

Summary. The data indicate that agglutination of sheep erythrocytes by influenza virus (WS strain) is influenced by physical-chemical factors within the test systems, and especially by the hydrogen-ion concentration. Over a relatively wide pH range, agglutination of sheep cells is inhibited by substances present in allantoic fluid, normal and infected. The allantoic fluid inhibition is ineffective in the pH range 5.8 to 6.0. When the test systems are adjusted with pH 5.8 buffer, and ½% to ¼% cell suspensions are employed, sheep erythrocytes may be used with essentially the same results as obtained with chicken cells, for the determination of influ-

enza virus content of allantoic fluids, and for the demonstration of antibodies by the agglutination-inhibition test. The data are significant because of the bearing they have upon the mechanism of virus hemagglutination.

15992 P

Widespread Distribution of Poliomyelitis in Households Attacked by the Disease.*

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Among patients admitted to the University of Kansas Hospitals because of poliomyelitis in 1946 it was clear that intercurrent illness had occurred in additional family members. Several households were selected for study on the basis that other illnesses had occurred at a time when poliomyelitis attacked a member of the group. Each member was sampled to determine whether poliomyelitis virus could be found in the oropharyngeal exudate and intestinal effluvia. Results obtained in a study of 4 households are reported here.

Material and methods. Throat Swabs: Cotton pledgets on applicator sticks were used to sample exudate from the oropharynx of each person. Pledgets moistened with exudate were stored at -70°C in sterile individual screw-capped bottles containing 1 cc of distilled water. In the preparation of each specimen and its inoculation into monkeys we have used methods described in earlier papers.[1,2,3]

Stool Specimens: The manner of collection of stool samples has been described.[4] No more than 12 hours elapsed between collection of each specimen and storage in a dry ice chest. The method of preparation and inoculation of monkeys with each specimen has also been described.[5,6] Each stool sample was inoculated into a monkey using the intraperitoneal and intranasal portals.

Monkeys: Rhesus *(Macaca mulatta)* and cynomolgus *(Macaca irus)* monkeys were used. A positive test in this study indicates that the monkey developed paralysis. In addition lesions consistent with those observed in poliomyelitis were observed in tissues from the spinal cord and elsewhere in the cerebral axis.

Results. The results of tests for poliomyelitis virus in oropharyngeal exudate and feces derived from each member in each of 4 households appear in Table I.

Twenty people living in these 4 households were studied. Sixteen had poliomyelitis virus in their intestinal discharges, and among these 16, 7 had virus in the oropharynx. Of 12 children in these families, each had poliomyelitis virus in the stool specimen; 5 had virus in the oropharynx. Considering the 8 adults in these households, 4 had poliomyelitis virus in respective stool samples. Among these 4, virus was detected in the throat of 2 fathers. Poliomyelitis virus was not found in the remaining 4 parents.

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc., New York City.

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2 Howe, H. A., Bodian, D., and Wenner, H. A., *Bull. Johns Hopkins Hosp.*, 1945, **75**, 19.

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5 Trask, J. D., Vignec, A. J., and Paul, J. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 147.

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TABLE I.

Tests for Poliomyelitis Virus in Feces and Oropharyngeal Exudate in Members of 4 Households Attacked by Poliomyelitis.

Family		Age, yrs	Type of illness	Onset of illness, 1946	Feces			Oropharyngeal exudate		
					Collected	Monkey No.	Result	Collected	Monkey No.	Result
H.M.	♂	31	Abortive	June 20	June 26	R-116	+	June 26	C-129	0
	♀	37	Indefinite	22	25	R-122	0	25	R-84	0*
	♂	8	Abortive	17	25	R-113	+	25	R-98	0
	♂	6	Paralytic	18	24	R-90	+	27	R-85	0
	♂	2½	Abortive	17	25	R-117	+	27	R-96	0*
	♀	7 mo	”	15	25	R-115	+	27	R-101	0*
T.P.	♂	43	Indefinite	July 10	Aug. 3	R-119	0	Aug. 3	C-128	0
	♀	41	None	—	4	R-125	0	3	R-103	0*
	♀	11	Paralytic, mild	25	8	R-120	+	3	R-88	0
	♀	10	” fatal	23	2	R-89	+	July 26	R-86	+
	♀	4	None	—	2	R-112	+	Aug. 3	R-102	0*
	♂	3	Abortive	28	4	R-91	+	3	R-87	0
C.D.	♂	28	None	—	Aug. 4	R-121	+	July 31	C-130	+
	♀	28	”	—	July 31	R-124	+	31	R-104	0
	♂	6	Paralytic	25	Aug. 4	R-109	+	26	R-99	+
	♀	2	Abortive	29	4	R-111	+	31	R-94	+
R.C.†	♂	36	Abortive	?Aug. 5	18	R-118	+	Aug. 19	C-132	+
	♀	34	None	—	18	R-123	0	19	C-131	xd3
	♂	7	Paralytic	Aug. 11	18	R-110	+	17	R-97	+
	♀	6	”	13	18	R-114	+	17	R-95	+

0* = susceptible on challenge to local heterologous strain isolated during same outbreak.
x = incompleted test.
d = death.
3 = 3rd day.
R = *M. mulatta*.
C = *M. irus*.
† There is doubt about the accuracy of onset of illness in this patient.

TABLE II.
Comparative Data on Intestinal and Pharyngeal Specimens Obtained from Members of 4 Households Attacked by Poliomyelitis.

Type of illness	No. of cases	Mean interval in days between onset and sampling		Positive tests	
		Feces	Oropharynx	Feces	Oropharynx
Paralytic	6	8.6	5.3	6/6*	4/6
Abortive	7	8.3	8.3	7/7	2/7
Indefinite	2	14.0	14.0	0/2	0/2
None	5	—	—	3/5	1/4
	20			80%	37%

* Numerator—positive tests, denominator—individual tests completed.

Some facts concerning the members of these 4 households appear in Table II. The positive stool tests, aside from bringing persons who had poliomyelitis into view, confirm earlier studies.[7,8] Obviously it has been easy to detect poliomyelitis virus in stools. It is somewhat harder to find it in the oropharynx. If the detection of poliomyelitis virus in the throat has been irregular the results have a pattern. In other words it is not difficult to detect poliomyelitis in the oropharynx of a sick patient if sampling is done during the first few days of illness. It is harder to obtain specimens at an optimal period (1 to 5 days) in abortive and subclinical attacks of poliomyelitis unless symptoms occur at the same time or follow onset of illness in a recognized case in a household.

In reference to onset of illness (Table I) in members of these households it is evident that poliomyelitis virus had seated itself in individuals within a brief period (average 3.1, range 1 to 7 days). Illness if it occurred in additional family members preceded or closely followed the onset of symptoms in the recognized case.

Comment. It is quite clear that virtually all members in these households had poliomyelitis. As others[8,9] have, we found that adults as well as children had subclinical poliomyelitis. The detection of virus in 3 of 4 fathers is noteworthy, particularly in view of finding it in only one mother.

Our data are not extensive enough to determine how infection entered into the group. If the virus was introduced into the household by a carrier such an individual has been lost among other members who were found to harbor virus in the throat or stool. Indeed, there is suggestive evidence, based on the history of onset of illness, that infection occurred in these households as a result of exposure at a common source. Such an exposure must have been of short duration and taken place in a brief period of time.

Summary. A study of 4 households attacked by poliomyelitis provided evidence of widespread distribution of virus in the members of these units. There were 20 members in these households; 16 had poliomyelitis virus in their intestinal discharges; 7 had virus in the oropharynx.

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15993

Effect of Hypothyroidism and Hyperthyroidism on Mammalian Skeletal Muscle.*

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Observations on clinical hyperthyroidism frequently mention as part of the syndrome asthenia, dystrophy and fine tremors of voluntary muscle. Remission of some of these symptoms often follows thyroidectomy. Pathological changes have been observed in such muscles.[1] Creatinuria is often present,[2] further indicating the existence of some muscular disorders. The present study reports the effect of hypo- and hyperthyroidism on the weight, strength and fatigability of skeletal muscle of the albino rat.

Methods. A total of 150 albino rats closely matched as to initial body weight, age and sex, was used in this study. One group of adult rats was made hypothyroid by including thiouracil in the diet[3] in amounts sufficient to lower the B.M.R. by approximately 20% (2 g thiouracil per kg powdered Purina Laboratory Chow). The hyperthyroid state was produced in one group by subcutaneous injection of 1.5 to 2.0 mg thyroxin twice a week. A third group served as untreated controls. Treatment was carried out for an average of 60 days before measurements were made of muscle weight and strength. The strength of the intact gastrocnemius-soleus muscles was determined by measuring the maximal isometric tension response of the muscles to slightly supermaximal condenser discharge stimuli applied to the tibial nerve with the animal under light ether anesthesia. The technics that were employed for strength measurements have been described in detail elsewhere.[4]

For the fatigue studies 35-day-old rats were divided into 3 groups. One group was given subcutaneous injections of thyroxin twice a week in amounts of 6 mg per kg body weight. A second group was reared on the thiouracil diet and a third group served as their untreated controls. Fatigue studies were made 90 days later on these animals while they were under light ether anesthesia. This was done by optically recording the isometric tension response of the gastrocnemius-soleus group to condenser discharge stimuli at slightly supermaximal intensity applied at the rate of 120 per second to the tibial nerve and induction shocks applied directly to the muscles at the rate of 120 per second. The records were analyzed for 2 indices of fatigue. The tensions maintained at 5 and 10 seconds after the onset of stimulation were calculated as per cent of the earlier maximal tensions and the area under the first 10 seconds of the tension curve was measured planimetrically from optical records.

Results. No significant difference was found (Table I) between the weight and strength of control and thiouracil treated animals. The thyroxin treated animals exhibited muscular weakness. This was shown by the findings of lower total tension, reduced tension per g of muscle, less tension per unit of body weight and a slightly lower ratio of muscle weight to body weight. Similar results were obtained in the studies on the younger group of animals which were under treatment for longer periods of time. Isometric tetanus tension elicited by nerve stimulation declined more rapidly in the hyperthyroid animals than in their controls. This was evidenced (Table II) by a reduction in the area of the tension curve and by a

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2 Palmer, W. W., Carson, D. A., and Sloan, L. W., *J. Clin. Invest.*, 1929, **6**, 597.

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4 Hines, H. M., Thomson, J. D., and Lazere, B., *Am. J. Physiol.*, 1942, **137**, 527.

TABLE I.
Average Values, with Standard Errors, for Effect of Thyroxin and Thiouracil on Weight and Strength of Muscle.

	Control	Thyroxin	Thiouracil
No. of animals	54	45	51
Body wt before	173	171	170
'' '' after	269	240	248
Muscle* wt / Body wt × 100	0.650 ± 0.007	0.622 ± 0.007	0.676 ± 0.008
Total muscle* strength (g isometric tension)	3245 ± 84	2599 ± 149	3098 ± 90
Tension per g muscle*	1825 ± 35*	1629 ± 33	1806 ± 33
Total muscle* tension / Body wt	11.87 ± 0.253	10.26 ± 0.268	12.12 ± 0.162

* Gastrocnemius-soleus.

TABLE II.
Average Values, with Standard Errors for Area Under Fatigue Curve, and Per Cent of Maximal Tension Remaining at 5 and 10 Seconds Through Direct and Indirect Stimulation.

	Area under 10 second fatigue curve muscle activated through		Nerve stimulation % max. tension		Muscle stimulation % max. tension	
Treatment	nerve cm^2	muscle cm^2	5 sec %	10 sec %	5 sec %	10 sec %
Thyroxin	50.7 ± 1.80	82.0 ± 2.24	27.9 ± 0.97	11.3	78.3	50.9
Control	75.4 ± 1.81	106.1 ± 2.27	44.6 ± 1.15	14.1	75.9	51.6
Thiouracil	63.3 ± 2.24	104.0	36.4 ± 1.54	11.8	76.8	53.8

lower per cent of tension existing after 5 seconds of stimulation. The thiouracil treated animals were inferior to their controls in respect to the maintenance of tension in muscle during motor nerve stimulation. No significant differences were noted between the three groups for the per cent of maximal tension decreases after 5 seconds of direct muscle stimulation. However, the tension area for direct muscle stimulation was significantly lower in the thyroxin treated than in the control animals.

Discussion. The evidence on the whole indicates that the hyperthyroid state is associated with some degree of muscular weakness and increased susceptibility to fatigue. The failure to maintain tetanus tension from nerve stimulation together with normal response to direct stimulation suggests that the factor of neuromuscular transmission may be involved. The hypothyroid condition resulting from thiouracil administration was accompanied by normal muscle weight and strength relationship except for a slightly more rapid decline in the tetanus tension elicited by motor nerve stimulation. It is to be pointed out that these studies deal only with the capacity of the muscles to develop and maintain tension in response to nerve stimulation and are not concerned with the stimulus pattern that is utilized in the natural activation of the muscles in reflex and voluntary activity. The asthenia that might result from subnormal activation would not be apparent in this type of study.

Summary. Albino rats were made hypothyroid by treatment with thiouracil and hyperthyroid by administration of thyroxin. After 60 days of treatment, gastrocnemius-soleus muscles of throxin treated animals showed weakness and increased susceptibility to fatigue through motor nerve stimulation. Thiouracil treatment for a like period of time resulted in no loss of muscle weight or strength, but did produce a slight increase in susceptibility to fatigue by indirect stimulation.

15994

Further Observations on the Reproduction of Bacilli from Large Bodies in Proteus Cultures.*

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In a previous paper[1] the question was raised as to whether certain phenomena observed in Proteus may be sexual, in spite of the fact that fusion of cells is not involved. The experiments described in this note were undertaken to study this question. No evidence was obtained to prove that the influence of Proteus strains on each other is sexual, but the observations give further information concerning the remarkable antagonism between the strains.

The spreading filaments of Proteus when they meet filaments of another appropriate strain go through a peculiar transformation. They develop into large, round bodies, which continue to enlarge and reproduce either the usual small bacilli or tiny pleuropneumonia-like colonies. The influence of the spreading filaments on each other is quite specific. The usual small bacillary forms of Proteus are neither influenced by, nor exert any influence on, the filaments or small bacillary forms of another strain. The strains survive and retain their identity indefinitely in mixed cultures in broth. The motile filaments, so sensitive toward other Proteus strains, grow without hindrance through the various bacterial and mold colonies which they may encounter on the plates. The filaments are transformed into large bodies by relatively slight physical or chemical injuries; for example, refrigeration and exposure to tap water. The small bacillary forms are not sensitive to these influences. This reaction of the filaments may be accidental. The spreading filaments develop only on a suitable medium and only have a short existence before they break again into small bacilli. It is unlikely that during the brief period of spreading they are exposed to adverse conditions which would change them into large bodies. Hence, the formation of large bodies at the contact zone of two spreading strains may be a natural function of the filaments.

Although the usual mechanism of sexuality, the fusion of cells, is absent in these processes, it seemed desirable to determine whether the essential purpose of sexuality, the crossing or segregation of properties, is not accomplished by some other unknown mechanism. To study this question large bodies were isolated with a micromanipulator from the contact line of two spreading cultures, and their descendants were compared with the parent strains. The technique used for the isolation of the large bodies was similar in principle to that described by Dickinson.† The large bodies were transferred to small pieces of transparent agar, and their development into bacterial colonies was observed under the microscope. This procedure assured that the colonies developing on the agar were the descendants of the large bodies. From single colonies of each culture so obtained, several strains were cultivated for further study.

These strains were studied with respect to their serological properties and their antagonism toward other Proteus strains. Cultures from different Proteus strains planted beside each other on a plate do not cover the agar entirely, but a sharp line of demarcation re-

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1 Dienes, L., Proc. Soc. Exp. Biol. and Med., 1947, **64**, 165.

† See article of Stoughton.[2]

2 *System of Bacteriology*, London, published by His Majesty's Stationery Office, 1931, **9**, 105.

mains between the areas covered by different strains. Descendants of a single strain do not show this phenomenon. Approximately 70 strains isolated from different sources were examined in this respect. All combinations of these strains which were tried were antagonistic to each other. This antagonism between the strains offered a simple method to determine whether all descendants of a large body are similar and correspond to one or the other of the original strains. If the cultures are thinly planted and begin to grow in isolated colonies, even a single colony with strain specificity different from the others is clearly apparent in the plates.

Fifty-six large bodies were isolated from different combinations of 5 strains. Thirty-four of these developed into bacterial colonies and 18 did not grow. Their descendants were always similar to one of the original strains both in strain antagonism and serological properties. Neither segregation of properties nor crossing over from one strain to another was observed. However, it is of interest that with a single exception, in the combination of two strains, only large bodies belonging to one strain grew into bacillary colonies. For example, 34 large bodies were isolated from the combination of strain No. 3 with other Proteus strains. Fourteen large bodies did not grow. The 20 which did develop never reproduced strain No. 3 but always the other strain. Large bodies produced in strain No. 3 by refrigeration are fully viable. Twelve large bodies were isolated from the mixture of strains Sm and No. 14, both of which dominated over strain No. 3. Six large bodies were viable all of which corresponded to strain No. 14. In a combination of strains No. 14 and No. 52, 6 large bodies were isolated; 3 of these developed colonies corresponding to strain No. 52, one to strain No. 14 and 2 did not grow out. Strain No. 14 dominated over strain No. 3 and strain Sm, and was apparently equal to or weaker than strain No. 52.

The dominance of one strain over the other is not genetic, but it is probably the result of the strain antagonism already discussed. When the strains come in contact, both respond by transformation into large bodies, but apparently only the large bodies of one strain survive. The numerical relationship observed between the viable and non-viable large bodies may be influenced by the fact that large bodies are not produced in equal numbers by the different strains. Also, some are destroyed by the manipulation necessary for their isolation.

The observations in their present status give no information as to the function of the large bodies or the reproductive processes connected with them in Proteus. It is possible that their real significance is in connection with pleuropneumonia-like colonies and not with the direct reproduction of bacilli. The antagonism which appears in certain phases of growth between Proteus strains has thus far not been observed in other species.

Summary. Single large bodies were isolated from the contact line of 2 spreading Proteus strains. Their descendants were similar to one of the strains with respect to the properties studied, and crossing or segregation of properties was not observed. With a single exception, only one strain was recovered from the large bodies formed at the contact of 2 strains.

15995 P

Inhibition of Mumps Virus Multiplication by a Polysaccharide.

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Recently it was reported[1] that polysaccharides derived from various sources inhibit the multiplication of pneumonia virus of mice (PVM) in the mouse lung. The capsular polysaccharide of type B Friedländer bacillus proved to be the most effective of the preparations studied. Oxidation of the carbohydrate with periodic acid did not diminish its virus inhibiting activity, but the aldobionic acid derived by acid hydrolysis was without activity.

The effect of the Friedländer polysaccharide on the multiplication of viruses other than PVM has now been more extensively studied in the chick embryo. It has been found that the carbohydrate inhibits multiplication of mumps virus in the embryo, but does not inhibit multiplication of influenza A, influenza B or Newcastle disease viruses. Moreover, the polysaccharide inhibits hemagglutination of chicken erythrocytes by mumps virus, and prevents adsorption of the virus to such cells.

Methods. The Friedländer polysaccharide (Fr.B,) used in these studies was prepared and purified as described previously.[2,3] The viruses employed were: influenza A, PR8 strain; influenza B, Lee strain; mumps; Newcastle disease. They were maintained by passage in the allantoic sac of chick embryos. Allantoic fluid was used as a source of virus for infectivity and hemagglutination experiments.

Experimental. As a routine, 10% chicken erythrocyte suspensions were treated with 5 mg per cc of polysaccharide for 3 hours at room temperature. Chicken erythrocytes adsorb the polysaccharide, and it is not removed by repeated washing. Such treated red blood cells are neither agglutinated by mumps virus nor are they capable of adsorbing the virus as is shown in Fig. 1. Treated cells are partially or completely inagglutinable by influenza A, influenza B and Newcastle disease viruses. However, these viruses are adsorbed by and eluted from treated erythrocytes just as they are with untreated red blood cells. The results of a representative experiment with influenza B virus are shown in Fig. 1. The polysaccharide has no demonstrable direct effect *in vitro* on any of the above viruses, including mumps.

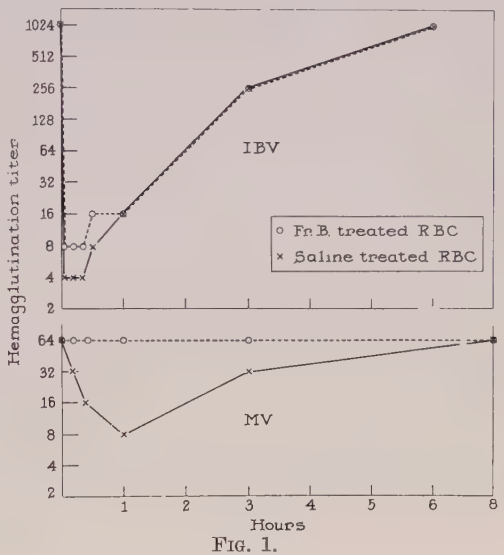

FIG. 1.
Comparison of the capacity of influenza B virus (IBV) and mumps virus (MV) to be adsorbed by and to be eluted from chicken red blood cells treated with Friedländer polysaccharide (Fr.B.).

When 0.5 to 1.0 mg of polysaccharide is injected into the allantoic sac of the chick embryo, a marked inhibition of the multiplication of mumps virus occurs even if the infecting dose is as great as 10^4 embryo infectious doses as is shown in Table I. Definite inhibition is obtained with as little as 50 μg of

1 Horsfall, F. L., Jr., and McCarty, M., *J. Exp. Med.*, 1947, **85**, 623.

2 Heidelberger, M., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1925, **42**, 701.

3 Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1927, **46**, 601.

TABLE I.
Effect of Friedländer Polysaccharide on the Multiplication of Mumps Virus in the Chick Embryo.

1st injection intra-allantoic 0.1 cc	Time between inj. hrs	2nd inj. intra-allantoic 0.1 cc	Mean hemagglutination titer‡ of allantoic fluids of embryos	
			Inj. with Fr.B. polysaccharide	Controls inj. with saline
Fr. B. polysaccharide*	3	Mumps virus 10^1 E.I.D.†	4	175
	3	10^2	1	264
	3	10^3	3	128
	3	10^4	4	245
Mumps virus 10^2 E.I.D.	3	Fr.B. polysaccharide	5	154
	24		2	154
	48		11	129
	72		240	—
	96		72	576
	120		384	—

* 1.0 mg per chick embryo.
† E.I.D. = chick embryo infectious doses.
‡ Mean of the reciprocals of the hemagglutination titers.

polysaccharide per embryo. In these experiments the concentration of virus in allantoic fluids was measured by the hemagglutination technique[4] 6 days following inoculation of the virus. As indicated in Table I polysaccharide may be injected 3 hours before, or as long as 48 hours after inoculation of mumps virus and still cause inhibition of multiplication of the virus. Multiplication of the virus in the allantoic sac is also inhibited when 5 mg of Fr. B. is injected into the embryonic yolk sac either 3 hours before or 3 hours after the inoculation of virus.

Comment. The role which the capsular polysaccharide of type B Friedländer bacillus plays in the inhibition of virus multiplication is not yet understood. Nor is it yet possible to correlate the activity of this and other carbohydrates with similarities in chemical constitution. It is possible, however, that knowledge of the biochemical mechanism involved in this phenomenon may lead to an understanding of the intracellular systems concerned in the multiplication of viruses. The fact remains that a substance belonging to a well-defined class of chemical compounds has been found which exerts specific inhibition on the multiplication of certain viruses in the living host.

Summary. The capsular polysaccharide of type B. Friedländer bacillus inhibits the multiplication of mumps virus in the chick embryo and prevents adsorption of the virus by treated erythrocytes.

[4] Levens, J. H., and Enders, J. F., *Science*, 1945, **102**, 117.

15996 P

An Improved Benzene Extracted Complement Fixing Antigen for Certain Neurotropic Viruses.*

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DeBoer and Cox[1,2] have recently described benzene-extracted mouse-brain and chick embryo complement fixing antigens for the diagnosis of neurotropic virus infections. The advantage claimed is that these antigens do not react with syphilitic sera that have been inactivated at only 60°C. We have now prepared antigens according to this new technic for Western and Eastern equine, St. Louis, Japanese B and Hammon-Reeves California viruses. Procedures were repeated several times for most viruses until several lots had been prepared. However, although the antigens completely fulfilled the criteria claimed by the authors, and represent an important advance, their sensitivity was slightly less than that of those we were accustomed to using (prepared by either the technic of Casals[3] or by a modification of that of Havens *et al.*,[4] centrifuging 10 per cent mouse-brain at 16,000 r.p.m.). Furthermore, the preparation described was prolonged (requiring about 4 days), and in our opinion unnecessarily dangerous (transferring and pulverizing powdered antigen from filter paper). We therefore undertook several experimental modifications of this technic. A more complete paper with extensive protocols is being published separately.

Preparation of Antigens. Mice are inoculated intracerebrally with a 10^{-2} or 10^{-3} dilution of virus. The mice, when moribund, are anaesthetized, bled to death and the brains removed. The brains are then ground in a Waring blendor in pyrogen-free, fractionally distilled water to make a 20% suspension. After storage for 3 to 4 hours at 5°C, 25 ml amounts are rapidly shell frozen in 250 ml Pyrex bottles and then lyophilized. The dried tissue is then extracted with benzene for 1 hour at room temperature by adding a volume of benzene equivalent to twice the original aqueous suspension. The benzene is rapidly removed by filtration through a Gooch crucible filter under high vacuum. Two further benzene extractions are performed in the same filter, allowing 30 minutes at room temperature each time before applying vacuum. The extracted tissue is then transferred under a hood by inverting the Gooch filter over a wide mouth stemless funnel placed in the neck of a 250 ml Pyrex bottle and then tapping the crucible filter gently till the dried powder falls free. The remaining solvent is removed by applying negative pressure to the bottle. Saline is next added in the amount of the original volume and rehydration permitted to occur overnight at 5°C, then after centrifugation for 30 minutes at 10,000 r.p.m. the supernatant is removed and Merthiolate is added to a final dilution of 1:10,000. The antigen is ready for immediate use, but for prolonged storage or shipment it is again lyophilized. The antigen is complete within 36 hours after killing the mice.

Characteristics of the Antigen. All tests have been made using essentially the technic described by Casals.[3] The liquid antigen,

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† Recipient of a predoctoral fellowship from the National Foundation for Infantile Paralysis, Inc.

1 DeBoer, C. J., and Cox, H. R., *J. Bact.*, 1946, **51**, 613.

2 *Ibid.*, *J. Immunol.*, 1947, **55**, 193.

3 Casals, J., *J. Exp. Med.*, 1944, **79**, 341.

4 Havens, W. P., Jr., Watson, D. W., Green, R. H., Lavin, G. I., and Smadel, J. E., *J. Exp. Med.*, 1943, **77**, 139.

prior to the final lyophilization has remained unchanged in all its characteristics when stored at 5°C for at least 6 months. The final, dry product goes into complete solution rapidly and requires no centrifugation before use. Its titer is the same as before lyophilization, and if any particular lot of antigen has a low titer, by adding only half the original volume of water to the powder the titer can be doubled. The reconstituted antigens have been tested after 3 months storage in the liquid state and have shown no decrease in titer or tendency to become anticomplementary.

These antigens when first prepared have infective titers of 10^{-3} or 10^{-4}. After a few weeks in the liquid state at 5°C they become practically nonvirulent.

No human serum, syphilitic or otherwise, among approximately 500 that we have tested has given a non-specific response after inactivation for 20 minutes at 60° C.

These antigens should be diluted further for routine use, for when concentrated, relatively low serum titers are obtained. The optimal dilution is determined by performing a combined, serial, two-fold immune guinea pig serum, and two-fold antigen titration. The highest dilution of antigen giving the highest serum titer is selected for use. This is usually a dilution of 1:4 to 1:16 of the antigen (1.25 to 5% brain) and represents about 8 to 16 antigen units. With guinea pig immune sera, antigens titer (in serial 2-fold antigen dilutions) from 1:64 or 1:128 (Western equine and California virus) to 1:256 or 1:512 (Japanese B, St. Louis and Eastern equine). At the optimal dilutions no overlapping can be demonstrated between any of our antigens and any heterologous hyperimmune sera, not even between Japanese B and St. Louis, although when an excess of antigen is used such overlapping is observed.

Human convalescent sera for Western equine infections have titered as high as 1:64 (original serum dilution) and for St. Louis and Japanese B (sera collected by W. M. H. from 1945 Okinawa epidemic) up to 1:512. Human convalescent sera give higher titers with these antigens than with any others we have ever prepared and there has been greater uniformity of titer between repeated preparations with the same virus. Moreover, these are the only antigens that have not given us occasional non-specific reactions with sera from febrile patients with other infections.

These antigens all show a tendency to protect complement during overnight incubation at 5°C and thus avoid certain difficulties shown by some other antigens when used with sera that are very slightly anticomplementary, though not detectably so in the anticomplementary control.

Summary. A time saving and safer procedure for preparing benzene extracted brain antigens is described. The resultant antigen is more sensitive and more specific than others previously tested by us. In its final form it is a stable, lyophilized product that can be easily shipped and can be used without centrifugation after rehydration.

15997 P

Excretion of Poliomyelitis Virus.*

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During the early stages of human poliomyelitis virus appears in the pharyngeal secretions and in the stools.[1,2,3,4] The experiments of Melnick[5] suggest that this phenomenon is excretory, since it occurs in the stools after parenteral inoculation, but little is known of the mechanism or pathways of excretion. The present study is part of an exploration into the mode of virus excretion.

Following an hypothesis proposed in another paper[6] and using monkeys, we have applied 20% virus (our *Cam* strain, 2nd-7th passages) for 10 minutes to the central end of the divided infraorbital branch of the right trigeminal nerve in the cheek ("nerve dip"), sedulously avoiding virus contamination of the skin and mucous membranes. Preliminary tests with rhesus (*M. mulatta*) showed that on the third day heavy lesions were usually present in the homolateral but not the contralateral semilunar (Gasserian) ganglion, while no more than minimal lesions were found, and then only occasionally, in the central components of the trigeminal system in the pons and medulla. At the same period, virus was detected in the homolateral but not in the contralateral ganglion. Virus was also found in pooled nasopharyngeal washings collected from the 2nd to the 5th day, inclusive.

With these preliminary data at hand, 4 cynomolgus monkeys (*M. irus*) of Philippine origin were similarly treated and materials collected and pooled serially on the second, third and fourth days after exposure, the animals being sacrificed on the fourth day. Nasopharyngeal washings were obtained by washing 0.85% NaCl solution through one nostril with a catheter and collecting the returning fluid from the other. This material as well as the stools and intestinal contents was concentrated by the technique described by one of us (R.J.S.)[7] and subinoculated intrathalamically into rhesus monkeys. The results are shown in Table I.

Discussion. In a previous paper[6] we have suggested on anatomical grounds the hypothesis that when poliomyelitis virus, which is known to be axonally conducted, has penetrated a body surface, entered the telodendria of a peripheral afferent neuron and ascended to the corresponding peripheral ganglia where it can multiply and infect adjoining neurons, it would then be in a position to spread not only centripetally to the CNS but also centrifugally back to the surfaces supplied by the same nerve system. This possibility arises because the peripheral neurons of most afferent nerves have T-shaped axons, one branch of which ascends to the CNS and the other is directed to the periphery. In the case of the trigeminal nerve, which was investigated in the present experiments, virus would thus be brought back by centrifugal spread to the terminal arborizations in the nasooropharyngeal mucosa, which are stated to end free on the surface,[8] whence it might be

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

1 Taylor, E., and Amoss, H. L., *J. Exp. Med.*, 1917, **26**, 745.

2 Paul, J. R., Salinger, R., and Trask, J. D., *J. A. M. A.*, 1932, **98**, 2262.

3 Howe, H. A., Bodian, D., and Wenner, H. A., *Bull. Johns Hopkins Hosp.*, 1945, **76**, 19.

4 Horstmann, D. M., Melnick, J. L., and Wenner, H. A., *J. Clin. Invest.*, 1946, **25**, 270.

5 Melnick, J. L., *J. Immunol.*, 1946, **53**, 277.

6 Faber, H. K., and Silverberg, R. J., *J. Exp. Med.*, 1946, **83**, 329.

7 Silverberg, R. J., *Science*, 1945, **102**, 380.

8 Schaeffer, J. P., *The Nose, Paranasal Sinuses, Nasolacrimal Passageways, and Olfactory Organ in Man*, P. Blakiston's Sons and Co., Philadelphia, 1920, p. 285.

TABLE I.
Distribution of Poliomyelitis Virus After Right Infraorbital Nerve Dip. Four Cynomolgus Monkeys. Cam Strain of Virus.

Days after inoculation	Nasopharyngeal washings	Stools	Semilunar ganglia		Washed pharyngeal wall
			Right	Left	
2	0	0	—	—	—
3	+*	0	—	—	—
4	+	+	+	0	0

* Non-paralytic case: cord histologically positive.
— Not tested.
\+ Clinically positive: cord sections positive.
0 Cord sections negative; symptoms absent or very questionable.

excreted and detected in the overlying mucus.

Our experiments tentatively confirm the hypothesis in the following respects: (1) ascent by centripetal spread to the Gasserian ganglion; (2) limitation of infection to the nerve system in question during the first 3 days; (3) excretion of virus in the appropriate area on the third day. Avoidance of primary exposure of the mucosa to virus rules out implantation of virus on the mucosal surface. Excretion by the lymphatics is highly improbable, since lymph flow is away from rather than toward the surface.[9]

The very early appearance of virus on the nasopharyngeal mucosae is noteworthy and interesting in reference to similar findings in the human disease,[1–3] as is the fact that it appeared a day later in the stools than in the nasopharyngeal washings, which might be explained on the basis of swallowing.

This work is to be repeated and expanded.

Summary. Poliomyelitis virus when applied to the central end of a divided branch of the trigeminal nerve in the cheek travels centripetally to the corresponding semilunar ganglion within three days. Centrifugal spread to the nasopharyngeal surfaces, which are supplied by the trigeminal nerve, was demonstrated by detection of virus in the nasopharyngeal washings on the third and fourth days. Virus was also found in the stools on the fourth but not on the third day, suggesting that it had been swallowed. It is suspected that excretion, like invasion, of poliomyelitis virus occurs through axonal channels.

9 Yoffey, J. M., and Drinker, C. K., *J. Exp. Med.*, 1938, **68**, 629.

15998

Prolonged Survival of Adrenalectomized-Nephrectomized Rats on a Low Potassium Diet.*

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Adrenalectomized animals are extraordinarily sensitive to all types of stress, including the metabolic derangements following nephrectomy. During the course of investigations on the relation of the adrenal cortex to urea synthesis, considerable difficulty was met in maintaining life in adrenalectomized-nephrectomized animals long enough for satisfactory study. The limited survival was not influenced by the use of liberal amounts

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of desoxycorticosterone acetate and saline for as long as seven days preoperatively. Durlacher and Darrow[1] have already shown that the survival of nephrectomized but otherwise normal rats may be significantly prolonged by preoperative potassium depletion by a low potassium diet. Since the adrenalectomized rat is particularly sensitive to potassium accumulation, such as occurs after nephrectomy, the effects of a low potassium diet on the survival of adrenalectomized-nephrectomized rats were investigated. It was found that not only was the survival time prolonged over that of adrenalectomized-nephrectomized rats on the chow diet, but it was also significantly extended beyond that of nephrectomized, but otherwise normal rats also on a low potassium diet.

Methods. Male rats of the Sprague-Dawley strain, weighing 170-200 g, were fed Rockland rat chow until the beginning of the experiment. For 3 days before operation the animals received the following diet: Casein (Technical), 180; sucrose, 570; peanut oil, 220; cod liver oil, 10; brewers' yeast, 20; bone ash, 20; sodium chloride, 5. This diet contains 1.4 mEq of potassium per 100 g,‡ as compared to 24.6 mEq per 100 g of Rockland rat chow. The animals were allowed free choice of water and 1% saline in their drinking fountains throughout the experiment. On the evening of the third day, all food was removed, and the animals were anesthetized by the intraperitoneal injection of 3 mg nembutal per 100 g of body weight. After operation, the animals were bled 2 to 3 times daily from the tail, 0.3 ml of blood being removed and used for the determination of urea nitrogen by the xanthydrol method of Engel and Engel.[2] Total urea synthesis, expressed as milligrams of nitrogen per 100 g body weight per hour was calculated from the rise of blood urea nitrogen, assuming the urea to be equally distributed throughout the body water, *i.e.* 75% of the body weight. Survival times were judged to be the last time the rats were known to be alive. Thus rats dying during the night were considered to have a shorter survival time than was actually the case. This error equally affected both adrenalectomized and control animals. The largest error introduced in this assumption was not more than 6 hours. Urea levels at the time of death were calculated by assuming no change in the rate of urea formation from the time of the last determination to the time of death. In several instances where samples were obtained within an hour or less of the time of death, this assumption was found to be valid.

Nephrectomies and adrenalectomies were performed simultaneously through bilateral subcostal incisions. In several animals, the adrenal area was examined histologically after death and the absence of adrenal rests demonstrated.

Results. Table I compares the survival time of nephrectomized-adrenalectomized rats on the chow diet and previously treated with 5 mg of desoxycorticosterone acetate for 3 days with those on the low potassium diet, and with nephrectomized rats with intact adrenals on the low potassium diet. It is apparent that preparation with the special diet prolonged the life of the adrenalectomized-nephrectomized rats to a significant degree ($p<0.01$).§ There was also a significant increase in the survival time of adrenalectomized rats as compared to those which had merely been nephrectomized ($p<0.02$). When Upjohn's aqueous adrenal cortical extract‖ was administered to the adrenalectomized rats at a dose level of 1 ml twice daily through the course of the experiment, the survival time was intermediate between the control and the adrenalectomized group, but was not significantly different from either group ($p>0.05$).

[1] Durlacher, S. H., and Darrow, D. C., *Am. J. Physiol.*, 1942, **136**, 577.

‡ The potassium analysis was performed by the Department of Biochemistry, Emory University.

[2] Engel, M. G., and Engel, F. L., *J. Biol. Chem.*, 1947, **167**, 535.

§ Fischer's tables of p were used in determination of statistical significance. A value of p equal to or less than .02 is considered significant.

‖ We are indebted to Dr. D. J. Ingle of the Upjohn Co. for generous supplies of aqueous adrenal cortical extract.

TABLE I.
Survival Times and Terminal Blood Urea Nitrogen Levels After Nephrectomy.

	No. of rats	Survival time, hr	Terminal blood urea nitrogen, mg/ 100 ml
Adrenalectomized-nephrectomized, chow diet*	3	13.5 ± 2.3†	—
Adrenalectomized-nephrectomized, low potassium diet	7	98.4 ± 5.2	240 ± 5.1†
Nephrectomized, low potassium diet	7	77.1 ± 5.5	329 ± 35.92
Adrenalectomized-nephrectomized, low potassium diet, adrenal cortical extract	7	89.1 ± 3.4	264 ± 17.4

* Treated with 5 mg desoxycorticosterone acetate for 3 days prior to nephrectomy.
† Standard error of the mean.

The relation of the survival time to the degree of uremia is also shown in Table I which records the blood urea nitrogen concentration of the rats at the time of death. It was found that the final urea concentration of the adrenalectomized animals was lower than that of the non-adrenalectomized group, even though the survival time of the adrenalectomized group was longer. Once again, the adrenalectomized group treated with adrenal cortical extract fell into an intermediate position. The difference between the adrenalectomized and control animals was significant ($p<0.01$); those between the group given adrenal cortical extract and the other two were not ($p>0.10$).

When the rate of urea synthesis in the nephrectomized rats was compared with that of the nephrectomized-adrenalectomized rats, as shown in Fig. 1, no difference was found initially, but as the experiment progressed increasing and significant differences were found. There was a significant rise in the rate of urea formation in animals with intact adrenals, but the apparent fall in the adrenalectomized animals' rate was not significant.

Discussion. By depleting rats of potassium, it has been possible to maintain life long enough to study their rate of urea formation after adrenalectomy and nephrectomy. Two methods of potassium depletion are available; the use of the low-potassium diet, and the administration of 2 mg of desoxycorticosterone acetate per day for periods of 3 weeks.[1] We have elected to use the special diet because it is more convenient and less expensive than is desoxycorticosterone. It is probable that the high mortality noted in the group of rats on the chow diet was a result of an inadequate preoperative preparation with desoxycorticosterone, since at this dose level the period of preparation was too short to effect potassium depletion.

The 3-day preparatory period we have used probably does not achieve a maximal protective effect, since Durlacher and Darrow's rats[1] prepared with a similar diet for 21 days survived an average of 112 hours as compared to 77 hours for nephrectomized rats with intact adrenals in our series.

Selye and Nielsen[3] have reported increased survival time in rats pretreated with 10 mg of desoxycorticosterone acetate for only 3 days before nephrectomy. In their series, however, the increased survival time may have been due to the functional adrenalectomy produced by such large doses of desoxycorticosterone. The rate of urea formation of the pretreated animals was lower than that of the controls, suggesting a general depression of adrenal cortical function in this experiment.

It is of interest that, after potassium depletion, adrenalectomized rats survive nephrectomy for longer periods than do rats with intact adrenals. This is the only instance of which we are aware in which the adrenalectomized animal is more resistant to a stress, *i.e.* nephrectomy and fasting, than is the animal with intact adrenals. It should be emphasized, however, that the adrenalectomized potassium-depleted rat is not more resistant to uremia, since the blood urea nitrogen level at the time of death was lower

[3] Selye, H., and Nielsen, K., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 541.

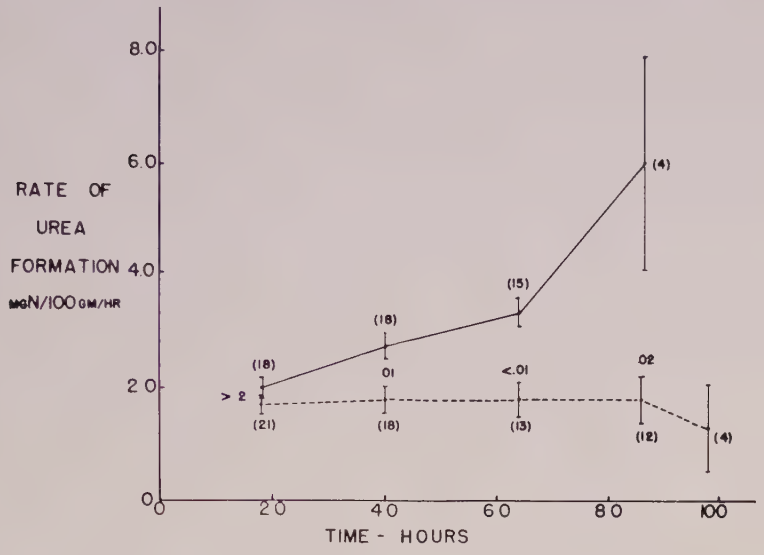

Fig. 1

The rate of urea nitrogen formation per 100 g body weight per hour after nephrectomy in the control (solid line) and adrenalectomized (dashed line) rats maintained on a low potassium diet for 3 days before nephrectomy. The numbers in parentheses refer to the number of observations at each point. The vertical bars represent the standard error of the mean of each point and the numbers between the curves the p values at each point.

in the adrenalectomized than in the control rats. The administration of adrenal cortical extract shortened the survival time of adrenalectomized rats. These effects can all be explained on the basis of the depression of protein turnover, and consequently, of urea formation observed after adrenalectomy. In the control group, the rising rate of urea production was probably due to the usual protein catabolic response to stress which occurs in the presence of the adrenal cortex. Harrison and Long[4] found that the rate of nonprotein nitrogen excretion increased after the second day of fasting in normal rats. The increasing difference in the rate of urea production between the adrenalectomized-nephrectomized and normal nephrectomized rats in our experiment is of the same degree of magnitude as found by Harrison and Long in their studies on nitrogen excretion. Thus starvation itself may be a sufficient explanation for the rising rate of urea production. The metabolic adjustments of uremia may play little or no part in accelerating urea formation in the nephrectomized rat with intact adrenals.

Summary. Adrenalectomized-nephrectomized rats fed a chow diet before operation have a high mortality rate. If the animals are prepared preoperatively for 3 days on a low-potassium diet, the adrenalectomized animals survive longer than rats with intact adrenals. The rate of production of urea is normal immediately after adrenalectomy, and fails to accelerate in the manner characteristic of rats which intact adrenals subjected to starvation and uremia. The blood urea nitrogen level at death is lower in adrenalectomized than in control animals. Administration of adrenal cortical extract to adrenalectomized-nephrectomized rats tends to restore the survival time and terminal blood urea nitrogen level to control values.

[4] Harrison, H. C., and Long, C. N. H., *Endocrinology*, 1940, **26**, 971.

15999

Attempts to Produce Acute Cardiac Failure by Posterior Pituitary Extracts.

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The counterpart of clinical cardiac failure which develops after prolonged left ventricular strain as a result of working against an elevated arterial pressure has probably not been produced acutely in animal experiments. Until this is accomplished the fundamental cardiodynamic mechanisms involved will not have been fully elucidated. It remains problematical, for example, whether the typical congestive heart failure which often develops clinically can occur as a direct consequence of prolonged strain of the left ventricle and reduction in its blood supply, or whether it is due to secondary causes. In the search for a method for producing hypertensive cardiac failure rather acutely, the use of posterior pituitary principles suggested itself. Extracts of the posterior pituitary gland containing chiefly or solely the pressor principles, when injected into anesthetized animals, cause an elevation of arterial pressure by peripheral constriction, constriction of coronary vessels of an intense degree, and probably as a result of such action depression of the myocardium. Pulsus alternans which becomes significant in view of the cardiac slowing induced has also been reported. According to Starling's conception, myocardial failure means dynamically that the ventricles require a greater diastolic distention and higher initial tension in order to expel the same stroke volumes. Indeed, it is by virtue of such depressive action on the ventricles that caution has been urged in the clinical use of posterior pituitary preparations.

Cardiac Effects from Continued or Repeated Administration of Posterior Pituitary Principles. Fourteen dogs weighing from 10 to 18.5 kilos were anesthetized with morphine sulfate and sodium barbital. Aortic pressure pulses were recorded by a calibrated Gregg optical manometer of adequate sensitivity and frequency. As a first approach, central venous pressures were measured by inserting a glass cannula into the superior vena cava via an external jugular vein and connecting it with the saline manometer, the pressure readings being made, after flushing, during expiration. In some experiments peripheral venous pressures were recorded as well. It was realized that this is a crude procedure and quite inadequate for dynamic studies, but it was sufficient to determine whether a pronounced rise of venous pressure such as is evident during clinical types of decompensation occurred. A number of different pituitary preparations were used and we were not able to detect any special difference between them.* In different experiments pituitary extracts were injected in two different ways, namely, by administration of fractional doses of 2 to 5 P.U. with an occasional administration of a larger dose and by slow continuous intravenous injection of a dilute solution over a period of hours. In the latter a solution containing 1 pressor unit per 10 cc was injected into a femoral vein by use of a Mariotte burette.

Results. Trials with different preparations revealed that a prolonged and marked hypertension cannot be realized from the use of pituitary extracts alone. This was due in a certain measure to the development of an apparent refractoriness to repeated injections of the extracts. Elevation of pressure to high levels was also prevented by the cardiac slowing which supervened and the myocardial depression which accompanied this slowing. However, at the moderate elevations of pres-

* Traveling Fellow of the Rockefeller Foundation.

† We are indebted to Parke-Davis and Co., Detroit; the Wilson Laboratories, Chicago; Eli Lilly, Indianapolis; and the Upjohn Company, Kalamazoo, for the large quantities of posterior pituitary preparations required in these investigations.

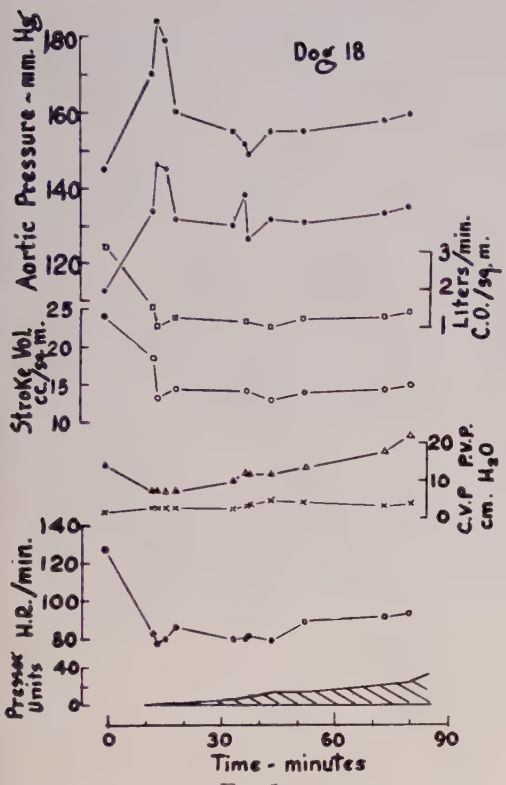

FIG. 1.

sures no consistent elevation of superior vena cava pressure was found to occur which in any way could be interpreted as congestive heart failure according to clinical standards.

Fig. 1 illustrates the main effects of a continuous accelerating infusion.‡ After a preliminary large rise of systolic and diastolic pressures to a maximum of 187/146 mm Hg (2 upper plots) these pressures decrease to somewhat lower ranges (average 155/132 mm Hg) owing partly to the marked retardation of the heart from 128 to about 80 beats per minute. Estimation of cardiac output by analysis of aortic pressure pulses after the method of Hamilton and Remington[2] indicates that the high arterial pressures are maintained despite the large reduction in stroke volume and cardiac output. Since the stroke volume is decreased despite the marked cardiac slowing it is apparent that the myocardium of the left ventricle is definitely depressed. This accords with observations previously reported by Wiggers[3] with regard to similar action on the right ventricle. Despite these changes, central venous pressure was not increased materially, although peripheral venous pressures measured by a catheter in the iliac veins rose moderately.

The nature of the myocardial depression induced by continuous action of posterior pituitary principles is revealed by a study of the aortic pressure pulses. These underwent changes in contour which were fairly constant and are exemplified by 8 segments in Fig. 2. Segment 1 is a normal control. Segment 2 shows an initial pressor effect, and segment 3 a subsequent depressor effect following an injection of 2 units of pitressin. Segment 4 represents the recovery but with the slowing maintained after this injection. Obviously, the effects of a single dose of posterior pituitary principles on rate persist longer than those causing depression of the myocardium. It is observed in segments 2 and 3 that regardless of whether the diastolic pressure rises or falls the pulse pressure is materially decreased. The isometric contraction phase indicated by the interval between the small preliminary vibration and the rise of the pressure pulse is definitely prolonged. Some recovery is noted in segment 4. The curve rises much more gradually to a summit in segments 2 and 3, indicating that the velocity of systolic discharge is reduced and its volume decreased. The magnitude of the change in stroke volume estimated according to the method of Hamilton and Remington is indicated directly on the record (S.V.). It will be noted that although the systolic discharge or stroke volume is restored in segment 4 the minute volume remains decidedly reduced owing to the continued reduction in heart rate (HR). Segments 5 to 8 indicate reactions during a later phase of the experiment after administration of pitressin had been discontinued for some time. Segment 5 shows some characteristics of pituitary action after another injection made after the curves of segment 4

‡ Indicated by cross-hatched lower graph.

2 Hamilton, W. F., and Remington, J., *Am. J. Physiol.*, 1947, **148**, 14.

3 Wiggers, C. J., *Am. J. Physiol.*, 1914, **33**, 382.

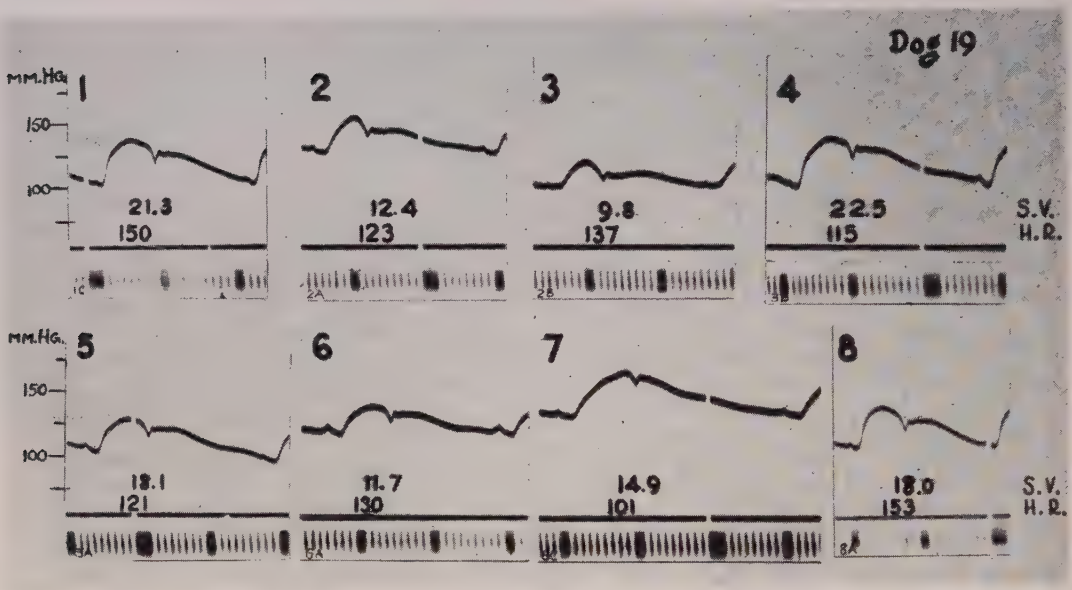

FIG. 2.

had been recorded. Segments 6 and 7 indicate additional and progressive effects of a continuous infusion. It will be noted that the stroke volumes and velocity of ejection by the left ventricle reduce significantly and that in segment 7 the stroke volume was diminished, although the pulse pressure was increased. Segment 8 shows considerable recovery, 52 minutes after the infusion was stopped.

Effects of Pituitary Extracts Plus Additional Hypertension. Since a sufficiently intense hypertension could not be produced by use of pituitary preparations alone, the technic was modified in two ways in an effort to make the left ventricle work against higher arterial pressure levels. In 4 dogs the carotid sinuses were excised and the vagi nerves cut. Thereupon, pitressin or pituitary extracts were again administered. Blood pressure was generally elevated at the start in such animals, although it tended to decline before pituitary extracts were administered. Progressive administration of pitressin either by continuous injection or given in stepwise doses failed to cause a pronounced elevation of arterial pressure required except very large doses which often proved treacherous. The observation was frequently made that after such deafferentation the heart rate, instead of decreasing, generally accelerated to an extreme degree. The mechanism remains enigmatic. The conclusion was reached that this also was not always a successful method for reproducing hypertensive cardiac failure acutely. A second modification consisted in opening the chest, maintaining a mild artificial respiration, and compressing the aorta to various degrees. This maneuver combined with injection of posterior pituitary extracts enabled us to produce higher systemic pressures although it involved certain complications as well. For example, too great compression of the aorta led to circulatory changes in the abdomen which tended to stagnate blood in the abdominal viscera. For this reason, such compression could not be maintained for too long a time. The typical responses following 3 periods of continued injection of pituitary extracts over a period of 3 hours are indicated in Fig. 3. Arterial pressures were maintained at rather high diastolic levels throughout, the heart rate became progressively slower, but central venous pressures remained remarkably constant. However, this may perhaps be accounted for in part by the reduction in venous return during aortic compression. Right ventricular failure in the clinical sense was

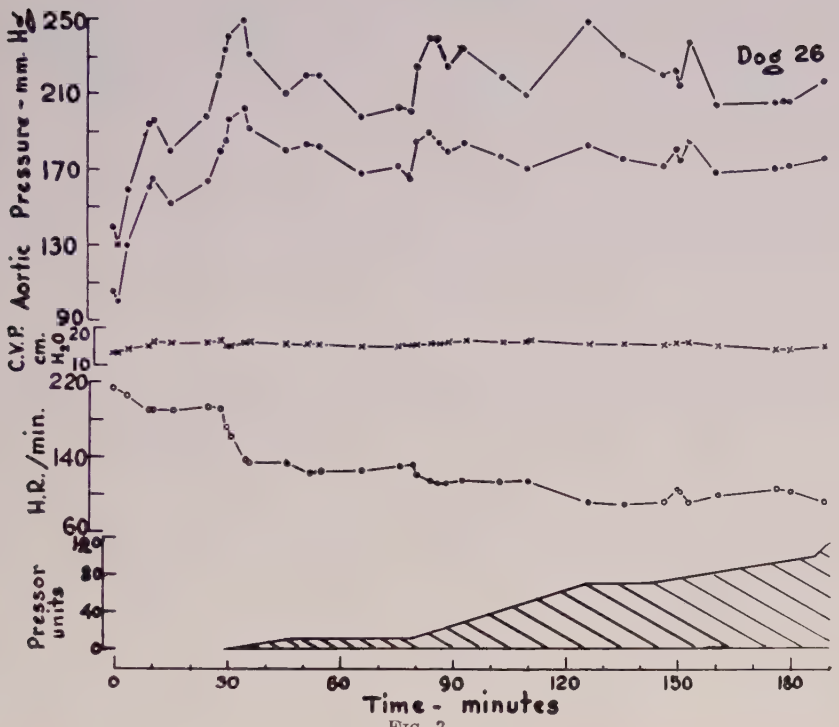

FIG. 3.

certainly not manifested in these experiments.

It was apparent after many trials that the congestive heart failure does not eventuate in dogs when a depressed left ventricle is required to work for 3 to 4 hours against high arterial resistance at the same time that the myocardium of the right heart is depressed and the coronary vessels are constricted. To assure ourselves that posterior pituitary preparations injected into a femoral vein is in sufficient concentration in the blood stream to reduce coronary blood flow while pressures are elevated, experiments were carried out in which flow in the left ramus descendens was measured either by the Opdyke flowmeter[4] or by a bubble flow meter. These experiments showed that significant reduction of blood flow occurred.

The conclusion was reached that if left ventricular strain accompanied by drastic reduction of coronary flow leads directly to cardiac decompensation the time factor must play an important role. Incidentally, our observations give some reassurance that while posterior pituitary preparations do induce coronary constriction and myocardial depression neither of these actions is apt to cause serious consequences when such preparations are administered in therapeutic doses to patients with normal coronary circulation and cardiac action. Careful continuous administration of 185 units during a period of 3 hours did not lead to permanent damage (Fig. 3).

Summary. An attempt was made to reduplicate clinical conditions in which a depressed left ventricle with a reduced coronary flow is required to work against high arterial pressures (a) by repeated or continuous infusions of posterior pituitary extracts, and (b) by creating additional arterial resistance through cutting of afferent moderator nerves or mechanical compression of the aorta. In all of these ways it proved impossible over periods of 3 to 4 hours to induce a state comparable to clinical congestive heart failure. If such failure is secondary to left ventricular strain plus reduction of coronary flow and

[4] Opdyke, D. F., and Foreman, *Am. J. Physiol.*, 1947, **148**, 726.

myocardial depression, the time element must play a dominant role.

Additional information regarding the nature of left ventricular depression induced by large doses of posterior pituitary extracts was obtained by an analysis of the aortic pressure pulses. Experimental evidence indicated that serious cardiac consequences are not apt to result from use of posterior pituitary preparations administered to patients with normal hearts in therapeutic doses.

We desire to express our appreciation to Professor C. J. Wiggers for his supervision of the experiments and help in interpreting the data.

16000 P

Effect of Estrogens on Early Development of Frog Embryos.*

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Boell[1] showed that cytochrome oxidase activity undergoes changes during the development of *Amblystoma punctatum* embryos, and McShan and Meyer[2] reported that certain estrogens inhibit the succinoxidase system in mammalian tissues. This inhibition by the estrogens was shown to be mediated through the cytochrome oxidase of the system. In the light of these results and the fact that cytochrome oxidase plays an important role in respiration, it became of interest to determine whether exposure of fertilized frog eggs to natural and synthetic estrogens would affect their normal development. Eggs of *Rana pipiens* and *Rana clamitans* were treated with diethylstilbestrol and with estrone in varying concentrations and for varying periods of time. The results of these preliminary experiments will constitute the data presented in this paper.

Frogs were procured from a dealer and stored in the laboratory at 4°C until used. Ovulation was produced and the eggs fertilized according to the standard technique of Rugh.[3]

A 10^{-3} M stock solution of diethylstilbestrol was prepared as reported by McShan and Meyer, and a solution of estrone of the same strength was made by dissolving 2.7 mg of estrone in 0.3 cc of 2 N NaOH plus 0.2 cc of 95% alcohol and diluting to a volume of 10 cc. The required strength of the estrogens was obtained by dilution with 20% Holtfreter's solution.[4] Proper control solutions of NaOH and alcohol were used.

The eggs were treated 1 hour after fertilization (after rotation and before the first cleavage) by dividing them into groups of 30 and placing them in 50 cc of the test solution. After exposure the eggs were rinsed with distilled water, transferred to Holtfreter's solution and maintained at 18°C for 5 days. Stages of development attained by normal and abnormal embryos were designated according to Shumway.[5] Abnormalities were recorded by number from Rugh's series of photographs.[3]

In the first series of experiments eggs of *Rana pipiens* and *Rana clamitans* were exposed to diethylstilbestrol in concentrations of 27, 13.5 and 6.8γ per cc for periods increasing by half hours from ½ hour to 4 hours. Control groups of eggs were placed in 6/100,000, 6/200,000 and 6/400,000 M NaOH and in 20% Holtfreter's solution. In

* Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

1 Boell, Edgar J., *J. Exp. Zool.*, 1945, **100**, 331.

2 McShan, W. H., and Meyer, Roland K., *Arch. Biochem.*, 1946, **9**, 165.

3 Rugh, Roberts, *Experimental Embryology*, 1941, N. Y. Univ. Bookstore, 18 Washington Place, New York.

4 Holtfreter, J., *Arch. f. Ent. Mech.*, 1931, **124**, 404.

5 Shumway, Waldo, *Anat. Rec.*, 1940, **78**, 139.

TABLE I.
Effect of Diethylstilbestrol and Estrone on the Development of Eggs of *Rana pipiens*.

Treatment* γ/cc	No. eggs	% abnormal	% survival
D 27.0	154	95.5	12.3
E 27.0	146	100.0	7.5
D 13.5	152	54.6	71.1
E 13.5	152	99.3	6.6
D 6.8	152	34.9	97.4
E 6.8	150	100.0	0
D 2.7	61	4.9	100.0
E 2.7	117	100.0	0.9
E 1.35	123	90.2	26.0
E 0.68	121	26.4	90.1

* Eggs were exposed to the solutions for a period of 2½ hrs beginning 1 hr after fertilization. Development was observed for 5 days.
D = diethylstilbestrol. E = estrone.

R. pipiens, with only one hour's exposure, 27γ of diethylstilbestrol per cc produced approximately 100% abnormal embryos and high mortality; 4 hours with 6.8γ per cc produced less than 40% abnormality and low mortality. 13.5γ per cc for 2½ hours, however, gave approximately 55% abnormality and 30% mortality. Eggs of *R. clamitans* showed a similar variation in response according to concentration and period of exposure but were more sensitive. 1 to 12% of control embryos were abnormal.

On the basis of these results the 2½ hour period of exposure was used for a comparison of the effects of estrone and diethylstilbestrol on eggs of *R. pipiens*. Concentrations ranging from 27 to 0.68γ per cc together with appropriate control solutions were employed. The data in Table I show that the effects obtained with estrone were much greater than those produced by diethylstilbestrol, and that when the ratio of abnormal embryos was about 50% or less the percentage survival was high. Abnormalities in the controls ranged from 1.3 to 10%. The stronger concentrations of both diethylstilbestrol and estrone produced a large number of abnormal embryos which did not develop beyond the blastula stage. Severe exogastrulation and various degrees of spinal bifida were the most common abnormalities observed.

Since it has been shown that estrogens inhibit cytochrome oxidase *in vitro*, further work is being done to determine whether this enzyme system can be inhibited in frog embryos by estrogens and thereby offer a possible explanation for the effects reported herein.

Summary. Abnormalities were produced in the developing eggs of *Rana pipiens* and *Rana clamitans* by exposure to diethylstilbestrol and to estrone in varying concentrations and for varying lengths of time. Higher concentrations of both substances produced more abnormalities, and estrone was found to be more effective than diethylstilbestrol.

16001 P

A Simple Method of Producing Control Guinea Pig Immune Sera for Use with Complement Fixing Antigens for the Arthropod-Borne Virus Encephalitides.*

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It was noted in this laboratory that the sera of guinea pigs, following the mild infection resulting from an intracerebral inoculation of St. Louis virus,[1] had good neutral-

* This investigation was carried out in collaboration with the Commission on Virus and Rickettsial Diseases, Army Epidemiological Board, Office of the Surgeon General, U. S. Army, and aided by a grant from the National Foundation for Infantile Paralysis, Inc.

† Recipient of a predoctoral fellowship from the National Foundation for Infantile Paralysis, Inc.

izing titers. Later, when complement fixation with this virus and the Japanese B virus was undertaken, it was noted that sera of guinea pigs recovering from infection with either virus, had moderate complement fixing antibody titers. Howitt[2] and later Brown[3] prepared such sera for equine viruses. However, most workers, following the recommendation of Casals and Palacios[4] and of Casals,[5] used hyperimmune mouse sera. Havens *et al.*[6] used hyperimmune hamster sera. Mice and hamsters have the unique advantage of being susceptible to fatal infections with all members of this group of viruses. Because of this, they can be given a long series of inoculations of any quantity of homologous brain-tissue virus. Disadvantages are that several months of immunization are required to obtain suitable titers; each animal when bled yields a very small quantity of serum, and because of anticomplementary or a non-specific type of reaction inherent in sera from these species, the sera require inactivation at temperatures of 60°C (mice) to 65°C (hamsters). Guinea pigs are susceptible to fatal infection only to the equine viruses. Using guinea pigs in long immunological procedures with mouse brain (other viruses) could be expected to result in sera reacting to all mouse brain antigens. Therefore, even though guinea pig sera require inactivation at only 56°C, they have been avoided in the past.

The following simple technic, however, produces specific sera of high titer in a very short period of time, with minimal work and with a relatively large yield per animal.

Western and Eastern Equine Viruses. 0.5 ml of a suspension of infected guinea pig brain of such dilution (10^{-2} to 10^{-4}) as will produce a paralytic, but non-fatal infection is given subcutaneously to each of a group of guinea pigs. Ten days later 0.5 ml of a 10^{-2} dilution is given intraperitoneally and after 7 to 10 days, 0.15 ml of a 10^{-1} dilution is given intracerebrally. After 2 weeks the animals are bled to death. The serum is pooled and frozen in CO_2 ice in a large number of small ampoules, or lyophilized in small tubes. The titer of this serum will fall quite rapidly when stored as a liquid at 5°C.

St. Louis, Japanese B, West Nile, Russian Spring-summer and Hammon-Reeves California virus. After a few serial passages in hamsters, ampoules of hamster brain suspension are frozen and stored in CO_2 ice. From the stock source guinea pigs are inoculated. Two intracerebral inoculations are given, 0.15 ml each, of 10% brain suspension at an interval of 10 days. Fever occurs in the guinea pig after the first injection, but rarely are other signs of infection observed. The animals are bled to death 10 or 15 days after the second injection, and the sera preserved as in the case of the equine viruses.

Characteristics of sera. These sera have been tested extensively over a period of several years with many types of brain antigen. A detailed report of the results is being published elsewhere. In no instance has a serum from any of these animals reacted with normal mouse-brain antigen, either before or after these immunization procedures. Japanese B and St. Louis sera usually have titers of 1:256, occasionally 1:512; Eastern equine sera are generally in the range of 1:128 to 1:256, while serum titers to the Western equine and California viruses are of the lowest order, usually 1:64 to 1:128. All are in a range where they can be used in relatively high dilutions as positive controls, or for the standardization of antigens.

When used with mouse-brain antigens made by the Casals technic or by our modification of that of Havens *et al.* (centrifuged at 16,000 r.p.m.), some minimal overlapping occurs between the Western and Eastern equine sera as reported for monkey and hamster sera by Havens *et al.*, and between the members of the St. Louis-West Nile-Japanese B complex. A slight but less pronounced over-

1 Hammon, W. McD., unpublished data.

2 Howitt, B. F., PROC. SOC. EXP. BIOL. AND MED., 1937, **35**, 526.

3 Brown, C. G., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 91.

4 Casals, J., and Palacios, R., *J. Exp. Med.*, 1941, **74**, 409.

5 Casals, J., *J. Bact.*, 1945, **50**, 1.

6 Havens, W. P., Jr., Watson, D. W., Green, R. H., Lavin, G. I., and Smadel, J. E., *J. Exp. Med.*, 1943, **77**, 139.

lapping occurs with sera of the latter group to the California virus antigen, but not in the reverse direction. With DeBoer and Cox[7] benzene extracted antigens, and our recent modification of their method,[8] much less overlapping occurs and when our antigen is employed in its optimal dilution (the highest antigen dilution which gives the highest serum titer in a combined antigen-serum titration), there is absolutely no overlapping detectable between any heterologous serum and antigen in original serum dilutions as low as 1:4.

Summary. Herein is described a simple, economical and quick method for preparing highly specific, high titer guinea pig immune sera for use as positive controls, and for standardizing antigens in the complement fixation test. This method has been used for producing sera for Western and Eastern equine, St. Louis, Japanese B, West Nile, Russian Spring-summer and the Hammon-Reeves California virus.

[7] DeBoer, C. J., and Cox, H. E., *J. Immunol.*, 1947, **55**, 193.

[8] Espana, C., and Hammon, W. McD., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 101.

16002

A Suitable Current Stabilizer for the Tiselius Apparatus.*

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It has become evident that present methods of current control for the electrophoretic apparatus of Tiselius can be improved. It is common practice to use a voltage-stabilized power supply to furnish the current. While this power supply gives a constant voltage across the whole cell, it does not furnish a constant current because of fluctuations of resistance within the cell. Most of these fluctuations probably arise within the electrode vessels. Inasmuch as the resistance of the central part of the cell is not a constant fraction of the total resistance of the cell, the voltage drop across this part and the field strength within it do not remain constant. Because of this it is customary[1] to measure the current and to calculate the field strength by means of the equation: $X = I/qk_s$.

X = field strength in volts per cm.
I = current in amperes.
q = cross-sectional area of the central part of the cell.
k_s = specific conductance of sample in reciprocal ohms.

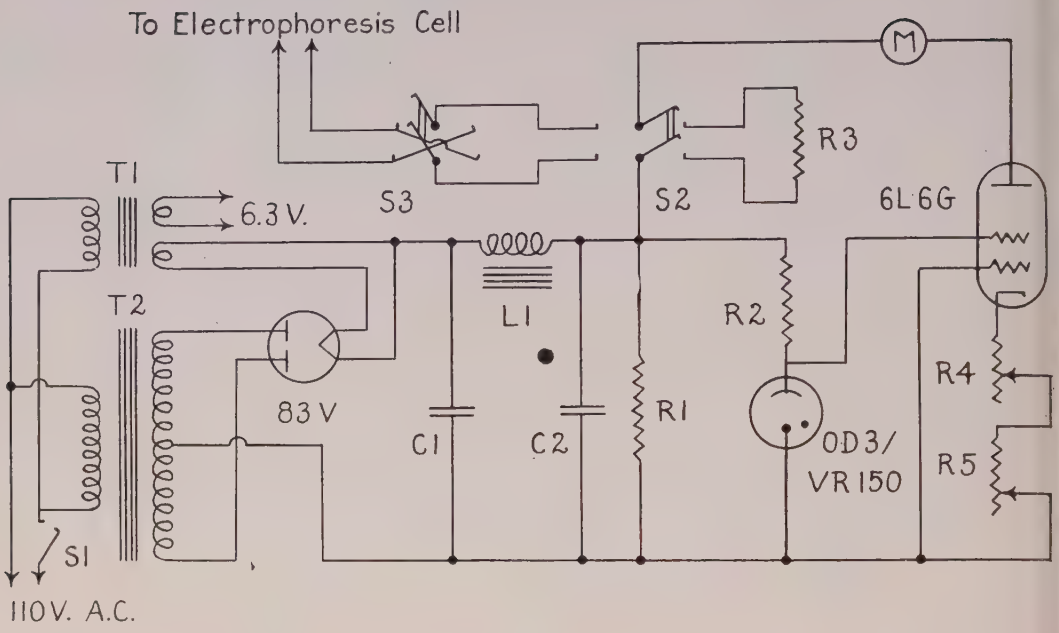
In order to apply this equation to apparatus employing a voltage-stabilized power supply it is necessary for the operator to make frequent observations of the current and to compensate for fluctuations by altering the applied voltage. This frequent adjustment is tedious as well as time-consuming.

It is preferable that current-stabilization rather than voltage-stabilization be used. The simple circuit which has been found suitable for the Tiselius apparatus is herein given. It is based on the principle that the plate current of a pentode vacuum tube remains constant despite changes in plate voltage. Further stabilization is obtained by the degenerative effect of the resistance in the cathode circuit.

In operation the switch S_2 is connected to the dummy load R_3 and then the switch S_1 is closed. This dummy load serves two purposes: first, the tubes are brought to normal operating temperatures in order to avoid subsequent fluctuations due to thermal changes; and secondly, the dummy load has approxi-

* Aided by a grant from the Commonwealth Fund. The materials needed for this investigation were supplied by the Charles F. Kettering Foundation.

[1] Abramson, H. A., Moyer, L. S., and Gorin, M. H., *Electrophoresis of Proteins*, p. 61, Reinhold Publishing Co., New York, 1942.



PARTS LIST

T1—Filament transformer with 5 V and 6.3 V windings.
T2—Power transformer—450 V each side of center tap.
L1—Filter choke—10 h.
C1, C2—Filter condensors—8 mfd, 600 V.
R1—50,000 ohms, 10 watts.
R2, R3—20,000 ohms, 50 watts.
R4—Potentiometer rheostat, 2000 ohms, 5 watts.
R5—Potentiometer rheostat, 200 ohms, 5 watts.
S1—S. P. S. T. switch.
S2, S3—D. P. D. T. switches.
M—0-30 milliameter, accurate to 0.5%.

mately the same resistance as the electrophoretic cell and so the 2,000 ohm rheostat R_4 may be set so that the current is approximately that required in the cell. Then S_2 is changed so that the current will flow through the cell and the final adjustment made with the 50 ohm rheostat R_5. S_3 is a reversing switch employed for alternation of the polarity of the electrodes on successive runs. The switches and controls are mounted so that the power switch S_1 is never closed when the cathode resistance is very small or when the plate circuit of the 6L6 G is open. This avoids damage to the tube.

It is found that the current variations with this source are undetectable on the milliammeter (½% accuracy) used to measure the current. The effect of fluctuations of line voltage and of cell resistance is negligible. Thus the current through the Tiselius cell is maintained at a constant value. The measurement of current by means of a standard resistance and potentiometer seems unnecessary and unjustified. A precise milliammeter is much more convenient and inexpensive and offers sufficient precision for all electrophoretic studies.

The cost of this circuit, exclusive of the milliammeter, was less than 20 dollars. For size, cost and performance it has been satisfactory for currents of 15-30 ma. It is possible that some of the simple constant-current circuits studied by Hill[3] could easily be adapted for use with electrophoretic cells.

[2] Swingle, S. M., *Rev. Sci. Inst.*, 1947, **18**, 128.

[3] Hill, W. R., *Proc. of the I. R. E.*, November, 1945, p. 785.

16003

Clotting Defect in Hemophilia: Deficiency in a Plasma Factor Required for Platelet Utilization.

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Previous studies[1] have shown that in hemophilic blood there is a delayed conversion of prothrombin into thrombin, apparently because liberation of thromboplastin from the formed elements of the blood is slow. Following removal of formed elements by centrifugation, normal plasma showed a delayed prothrombin conversion rate, but the delay was much less than in hemophilic blood. It was postulated that further centrifugation would produce in normal blood a slow clotting time and a delayed prothrombin conversion rate identical with those in hemophilic blood. It was suggested also that normal plasma, rendered spontaneously incoagulable by freeing it of its formed elements, was needed for a crucial test of the clotting defect in hemophilia.

Fuchs[2] reported having obtained spontaneously incoagulable human plasma by high speed centrifugation of carefully collected blood in paraffined glassware. Neither Feissly[3] nor Smith, Warner and Brinkhous[4] were able to confirm this work, despite the fact that equipment of Fuch's design was used. Recently Jaques and coworkers[5] reported the use of non-wettable surfaces treated with silicone to delay the clotting time of normal blood. In the experiments reported below this method has been adapted to obtain from normal individuals plasmas which clotted slowly or not at all in contact with *ordinary* glass. Such plasmas may be termed quasi-hemophilic since, like hemophilic plasma, they had a delayed prothrombin conversion rate but clotted promptly after addition of thrombin or thromboplastin. They were tested for their corrective effect on the clotting of true hemophilic blood and plasma. A preliminary report of these data was made recently.[6]

The technic used to obtain quasi-hemophilic and hemophilic plasmas was briefly as follows: Syringes, needles and glassware were treated with a methylchlorosilane, General Electric Dri-Film.[5] Blood was drawn from the antecubital vein directly into sodium citrate solution (3.2% $Na_3C_6H_5O_7 \cdot 2H_2O$) in the ratio of 8 ml blood to 1 ml anticoagulant. The blood was centrifuged initially up to 30 minutes in an angle centrifuge at 5700 r.p.m. (about 3300 g). The supernatant plasma then was subjected to a series of recentrifugations of 30-90 minutes each at the same speed. In instances in which the total centrifugation time exceeded 2 hours, the plasma was centrifuged for a period of 10-20 minutes at 14,000 r.p.m. (about 14,000 g) immediately after the initial centrifugation. Platelet-free plasmas were centrifuged for 165-1350 minutes, platelet-rich plasmas for 5 minutes. Collection and centrifugation of the blood were carried out in a constant temperature room (2°C).

Clotting time determinations were made after recalcification with $CaCl_2$ (1.2%) at 28°C in ordinary glassware. Each clotting tube contained 0.15 ml citrated plasma. Calcium, sufficient to give the shortest clotting time, and saline (0.9% NaCl) were added to make a total volume of 0.25 ml. In testing its effect on the clotting of hemophilic samples, the normal plasma was added in the ratio of 1 part to 10 parts hemophilic plasma. The

1 Brinkhous, K. M., *Am. J. Med. Sci.*, 1939, **198**, 509.

2 Fuchs, H. J., *Arch. exp. Zellforsch.*, 1933, **14**, 334.

3 Feissly, R., *Helv. Med. Acta*, 1940, **7**, 583.

4 Smith, H. P., Warner, E. D., and Brinkhous, K. M., unpublished data.

5 Jaques, L. B., Fidlar, E., Feldsted, E. T., and Macdonald, A. G., *Canadian Med. Assn. J.*, 1946, **55**, 26.

6 Brinkhous, K. M., *Fed. Proc.*, 1947, **6**, 389.

TABLE I.
Effect of Centrifugation Time on Clotting of Recalcified Normal Blood or Plasma.

Centrifugation time min.	Clotting time min.
0	6½
10	8½
30	10
60	18½
90	20
120	31
165	37
1350	no clot 1800

actual volume added was adjusted to correct for the citrate content. When whole blood was used, the volume was adjusted in addition for cell volume as indicated by the hematocrit.

Platelet suspensions were prepared by differential centrifugation of blood in silicone glassware. The platelet sediment was washed by resuspension in citrate-saline solution (1 part citrate solution, 9 parts saline) and recentrifuged. This was repeated 3 times. Microscopic examination showed the platelets to be well preserved. Platelet suspensions were added to provide 25,000-30,000 platelets per mm^3 in the final clotting mixture.

Most of the data on hemophilic blood was obtained from one patient, although the experiments were repeated on at least one of 3 other hemophilic patients. The normal blood samples were obtained from 7 different individuals.

Quasi-hemophilic plasma from normal blood. Table I shows that centrifugation of normal blood for ½ hour or less produced plasmas with clotting times within the range commonly accepted as normal, but that longer centrifugation produced progressively longer clotting times. Finally, plasma was obtained which did not clot within 30 hours after recalcification.

The prothrombin content[7,8] of several samples of quasi-hemophilic plasma, including those spontaneously incoagulable, was within the normal range of 90% to 110%. The prothrombin conversion rate of these samples, determined as previously described,[1] was delayed. The longer the clotting time, the greater was the delay.

Plasmas, similar to those shown in Table I, and whole blood were clotted with the same rapidity by thrombin solutions (Parke Davis "Thrombin Topical") and by thromboplastic extract of beef lung.[8] Tests for antithrombin activity gave normal values. All of the plasmas, when incubated with thrombin solutions, destroyed thrombin in the same amount and at the same rate.

The addition of platelet suspensions to the quasi-hemophilic plasmas reduced the clotting time to 6 to 9 minutes. This is in contrast to the behaviour of platelet-free hemophilic plasma described later (see Table III).

Failure of quasi-hemophilic plasma to correct the clotting defect of platelet-free hemophilic plasma (see Table II). Normal citrated whole blood reduced the clotting times both of whole hemophilic blood and of platelet-free hemophilic plasma to 6 minutes. The effectiveness of the normal plasmas in correcting the clotting defect of platelet-free hemophilic plasma decreased as their clotting times became prolonged. The spontaneously incoagulable normal plasma was entirely without effect. Regardless of the degree of prolongation of their clotting times, however, the normal plasmas retained their corrective action on the clotting of whole hemophilic blood.

Corrective action of normal plasma on the clotting of hemophilic plasma in the presence of platelets. In a series of further tests, quasi-hemophilic plasma was added to platelet-rich hemophilic plasma, and platelet-rich normal plasma was added to platelet-free hemophilic plasma. In the presence of numerous platelets, either normal or hemophilic, the hemophilic clotting time was reduced to the normal range. The results of one test follow. When a sample of platelet-poor normal plasma which had a clotting time of 27 minutes was added to platelet-free hemophilic plasma, the clotting time was 25 minutes; when it was added to platelet-rich hemophilic plasma the clotting time was 7 minutes, and when added to whole hemophilic blood, clotting occurred in 7½ minutes.

Effect of platelet suspensions on clotting of

7 Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

8 Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, **66**, 801.

TABLE II.
Effect of Addition of Normal Blood or Plasmas on Clotting of Hemophilic Blood or Plasmas.

Clotting time Recalcified normal plasma (to be added to hemophilic plasma), min.	Clotting time Recalcified hemophilic blood or plasma, after addition of normal plasma	
	Whole hemophilic blood, min.	Platelet-free hemophilic plasma, min.
6*	6	6
8	7	18
17	6½	19
31	6	26
40	7¾	30
no clot 1800	6	no clot 1800
	45-120†	no clot 1800†

* Whole blood.
† Controls, showing clotting times of various hemophilic specimens after recalcification, without normal plasma addition.

TABLE III.
Effect of Platelet Suspensions and Normal Plasma* on Clotting of Platelet-free Hemophilic Plasma.*

Supplement	Clotting time Recalcified hemophilic plasma, after addition of platelets or plasma, min.
Normal platelet suspension	32
Hemophilic platelet suspension	28
Normal platelet-poor plasma†	30
Normal plasma + normal platelet susp.	8½
Normal plasma + hemophilic platelet susp.	6

* Plasmas used in this experiment had been centrifuged for 120 minutes.
† Clotting time of recalcified normal plasma was 32 minutes.

platelet-free hemophilic plasma (see Table III). With the platelets alone the clotting time remained prolonged, but in the presence of normal plasma, which by itself did not correct the clotting defect, the platelets reduced the clotting time to the normal range.

Discussion. The data indicate that the clotting of normal plasma depends upon the presence of formed elements. Plasma deprived of formed elements or their disintegration products is stable, even in the presence of a wettable surface and optimal calcium in concentration. The ability to clot is restored by addition of platelets. Unless formed elements are present, normal stable plasma is without effect on the clotting of hemophilic plasma. These data indicate that platelets and perhaps other formed elements cannot be utilized in the absence of a plasma factor present in normal blood.

A simple hypothesis based on these findings is offered: Normal plasma contains a factor necessary for thromboplastin liberation from the formed elements of the blood, presumably by platelet lysis. This platelet-lysin or thrombocytolysin is deficient in hemophilic plasma. When the lytic factor is supplied to hemophiliacs, as in blood or plasma transfusions, platelets rupture in the normal manner. Sufficient thromboplastin then becomes available, and the block in the clotting mechanism is removed.

Numerous workers, including Feissly,[9] have attempted to test the effect of normal plasma on hemophilic plasma in the absence of platelets. Their results have shown an acceleration of the clotting of hemophilic plasma in the apparent absence of platelets. These discordant results may be due to the presence of free thromboplastin in the normal plasma from breakdown of the formed elements during collection and handling of the plasma, and perhaps also from incomplete removal of formed elements by centrifugation.

In the experiments presented in this paper, the centrifugal forces used were so low that it appears unlikely that any macromolecular substance essential for clotting, such as the thromboplastic protein described by Chargaff

[9] Feissly, R., *Helv. Med. Acta*, 1945, **12**, 467.

and West,[10] could have been removed by the centrifugation.

Summary. 1. By special handling and prolonged centrifugation of normal blood, plasmas with a delayed clotting time can be obtained. If centrifugation is prolonged sufficiently, the plasmas become spontaneously incoagulable.

2. Normal plasmas of this type require the presence of platelets and perhaps other formed elements to correct the delayed clotting of hemophilic plasma.

3. These findings indicate that in hemophilia there is a deficiency in a plasma factor required for platelet utilization. It is suggested that this factor is a thrombocytolysin.

10 Chargaff, E., and West, R., *J. Biol. Chem.*, 1946, **166**, 189.

16004

Effect of Penicillin on the Tubercle Bacillus *In vitro.*

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Reports concerning the effect of penicillin on the tubercle bacillus are contradictory. The Oxford group originally noted no inhibition of growth in glycerol broth with a penicillin concentration of 40 units per cc.[1] Subsequently, inhibition by as little as 20 units per cc was observed by one investigator,[2] whereas others reported no inhibition with concentrations of 20 to 30 units per cc.[3,4] Actual stimulation of growth by small concentrations of penicillin was described in one instance,[5] while in another, rapid destruction of large amounts of penicillin (800 units per cc) by culture filtrates of *M. tuberculosis* was observed.[6] These contradictory results, and the desirability of using penicillin to suppress the growth of contaminants in cultures for the primary isolation of tubercle bacilli, have made it appropriate to reinvestigate the effect of penicillin on the tubercle bacillus *in vitro*.

Materials. H37Rv, a standard virulent human strain of *M. tuberculosis,* was used throughout the experiments because of its similarity in virulence and response to antimicrobial agents to strains isolated from patients with tuberculosis.[7,8]

The experiments were performed in liquid and on solid media recently developed in this laboratory.[9,10] The penicillin employed was commercial crystalline penicillin G, obtained from various manufacturers.

Experimental. Lytic Action of Penicillin. A large inoculum of tubercle bacilli, 0.25 mg per cc, was added to test tubes 25 by 150 mm each containing 15 cc of Tween-albumin medium and penicillin in concentrations ranging from 100 to 1000 units per cc. The tubes were incubated at 37°C, and optical densities were recorded on the Coleman Spectrophotometer every day or two. Because of the possible destructive action of the tubercle bacillus on penicillin, the original concentrations were added to one set of tubes every 3 days on 4 occasions. To another set, no further penicillin was added to the amounts pres-

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1 Abraham, E. P., *et al., Lancet,* 1941, **2**, 177.

2 Hand, C. N., *J. Path. and Bact.*, 1946, **58**, 495.

3 Smith, M. I., and Emmart, E. W., *Pub. Health Rep.*, 1944, **59**, 417.

4 Friedmann, I., *Tubercle,* 1945, **26**, 75.

5 Ungar, J., and Muggleton, P., *J. Path. and Bact.*, 1946, **58**, 501.

6 Woodruff, H. B., and Foster, J. W., *J. Bact.*, 1945, **49**, 7.

7 Middlebrook, G., and Yegian, D., *Am. Rev. Tuberc.*, 1946, **54**, 553.

8 Feldman, W. H., and Hinshaw, H. C., *Am. Rev. Tuberc.*, 1947, **55**, 428.

9 Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

10 Dubos, R. J., and Middlebrook, G., *Am. Rev. Tuberc.*, in press.

ent at the beginning of the experiment.

Results of a representative experiment are presented in Table I. Concentrations up to 200 units per cc produced little or no inhibition of growth when no further penicillin was added. When penicillin was added at 3 day intervals, inhibition was noted in the tube originally containing 200 units per cc, indicating that penicillin inactivation was relatively slight, and that the final concentration was considerably in excess of 200 units per cc. With concentrations above 600 units per cc there was not only inhibition, but an actual decrease in optical density, indicative of lysis of the organisms.

A secondary outgrowth of the inhibited and partly lysed organisms was observed in the tubes containing 600 and 800 units per cc, when no more penicillin was added, but no secondary outgrowth occurred with a concentration of 1000 units per cc.

Relation of Inoculum Size and Medium to Penicillin Inhibition. Tubes containing 5 cc of liquid Tween-albumin or oleic acid-albumin media with various concentrations of penicillin were inoculated with tenfold dilutions of a 7-day-old culture of H37Rv diluted in 0.5 cc of distilled water. The inoculum for one set of tubes had been grown in Tween-albumin medium (dispersed growth), and for another set in oleic acid-albumin medium (granular growth). To allow for possible penicillin inactivation, 2 sets of tubes were inoculated. To one set, penicillin in the original concentrations was added on 3 occasions at 3-day intervals; to another no further penicillin was added to the amounts present at the beginning of the experiment. The results were recorded after 14 days incubation, complete inhibition of growth being regarded as the endpoint.

As shown in Table II, the inhibitory effect of penicillin was much greater in the Tween-albumin medium than in the oleic acid-albumin medium, and this difference was related to the size of the inoculum. In the oleic acid-albumin medium, growth was inhibited by 1000 units per cc; with 100 units per cc, no

TABLE I.

Bacteriostasis and Lysis of a Large Inoculum of Tubercle Bacilli by Penicillin in Tween-Albumin Medium.

A. Original Amounts of Penicillin Added 4 Times at 3-day Intervals.

Days	1	4	8	13	17
Penicillin concentrations, units per cc					
0	.082*	.156	.252	.366	.41
100	.082	.155	.222	.301	.40
200	.082	.125	.201	.275	.318
400	.082	.082	.061	.061	.061
600	.082	.061	.051	.051	.051
800	.082	.061	.051	.051	.051
1000	.082	.055	.051	.051	.051

B. No Penicillin Added to Initial Concentrations.

Days	1	4	8	13	17
Penicillin concentrations, units per cc					
0	.082*	.156	.252	.376	.43
100	.082	.156	.252	.376	.43
200	.082	.136	.252	.356	.41
400	.082	.102	.214	.346	.41
600	.082	.082	.175	.327	.41
800	.082	.061	.055	.097	.20
1000	.082	.055	.055	.055	.055

* Turbidities (optical densities) were measured with a Coleman spectrophotometer.

inhibition occurred, growth being as good as in the control tubes, regardless of the size of the inoculum. With the Tween-albumin medium, on the other hand, growth in 100 units per cc occurred only with the largest inoculum, and with smaller inocula the amount of penicillin necessary to suppress growth rapidly declined to 1 unit per cc or less.

When the inoculum was grown in the oleic acid-albumin medium, *i. e.*, when it consisted of clumps rather than diffusely growing bacilli, the inhibitory effect of penicillin in the Tween-albumin medium was somewhat less marked. This suggests that the clumps were better able to survive initially in the presence of penicillin. However, the Tween effect was not overcome entirely, possibly because even with clumps as an inoculum, subsequent growth in the Tween-albumin medium was diffuse.

As in the previous experiment, evidence of destruction of penicillin by the tubercle bacil-

lus was slight. Except for the first two tubes in the oleic acid-albumin medium, the end-point was the same whether further penicillin was added or not.

These results were extended to the solid oleic acid-albumin agar medium, on which, even with the smallest inocula (3 or 4 organisms per plate), no inhibition of growth was noted with penicillin in a concentration of 100 units per cc.

In preliminary experiments with contaminated material, quantitative plate counts were made from saline suspensions of the macerated lungs of Swiss mice infected intravenously with H37Rv. The control plates were often so heavily contaminated with other bacteria that the presence or absence of tubercle bacilli could not be determined. Plates containing penicillin in a concentration of 100 units per cc almost invariably showed complete suppression of growth of the contaminants, and in no instance was there evidence of inhibition of growth of the tubercle bacilli.

Comment. The foregoing experiments demonstrate that under appropriate circumstances tubercle bacilli undergo partial lysis in the presence of penicillin. Many other bacteria are lysed by penicillin, some, such as staphylococci, quite rapidly and completely,[11] and others, such as anaerobic streptococci, more slowly and to a lesser degree.[12] Tubercle bacilli appear to fall into the latter category.

The explanation for the much greater inhibitory effect of penicillin in the Tween-albumin than in the oleic acid-albumin medium is not entirely clear, but it may be found in part in the more intimate contact between the antibiotic and the individual organisms in the Tween-albumin medium, in which growth is diffuse, than in the oleic acid-albumin medium, in which growth is granular. In addition, specific alterations in the physico-chemical characteristics of the cell, caused by the presence of the non-ionic, surface active wetting agent "Tween 80," may play a major role in the phenomenon. Enhancement of the activity of antimicrobial

[11] Kirby, W. M. M., *J. Clin. Invest.*, 1945, **24**, 165.

[12] Todd, E. W., *Lancet*, 1945, **2**, 172.

TABLE II.

Inhibitory Effect of Penicillin on Various Inocula of Tubercle Bacilli in Tween-albumin and Oleic Acid-albumin Media.

Penicillin, units per cc	Inoculum grown in Tween-albumin medium										Inoculum grown in oleic acid medium									
	Penicillin added at 3-day intervals										No additional penicillin added									
	Tween medium					Oleic acid medium					Tween medium					Oleic acid medium				
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inoculum	*1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5

* 1—2.5-2 mg dry wt. 2—2.5-3 mg dry wt. 3—2.5-4 mg dry wt. 4—2.5-5 mg dry wt. 5—2.5-6 mg dry wt.

agents against other bacteria by various wetting agents is well known.[13] This increased inhibitory action of penicillin against the tubercle bacillus in the Tween-albumin medium is similar to that recently observed by Fisher with streptomycin.[14]

The relatively slight destruction of penicillin by the human virulent strain H37Rv in the present studies is in contrast to the marked destruction reported by others using nonpathogenic laboratory strains.[2,6] It may be noted that one of these strains, No. 607, which is often used in studies of tubercle bacilli, does not fulfill the cultural and biological requirements of a true tubercle bacillus. It should be stated that since there was relatively little decline in penicillin activity, no attempt was made in the present experiments to determine quantitatively the extent to which the decline was due to inactivation of penicillin by tubercle bacilli, to deterioration in the incubator, or to a secondary outgrowth of resistant variants.

It would appear that the addition of penicillin in concentrations of 50 to 100 units per cc to the liquid or solid oleic acid-albumin medium may prove a valuable adjunct in culturing contaminated materials.

Current methods of treating clinical specimens often do not destroy all of the contaminants, and inhibitory dyes, such as malachite green, which are ordinarily added to the classical egg yolk-potato media, may also inhibit the growth of the tubercle bacillus.[15] It should be realized, of course, that many types of organisms are not inhibited by penicillin, although concentrations as high as 50 to 100 units per cc may be adequate to suppress the growth of many bacteria and fungi not ordinarily considered susceptible.

Summary and Conclusions. Using appropriate experimental conditions, a virulent human strain of *M. tuberculosis* has been found to undergo partial lysis in the presence of high concentrations of penicillin.

Small inocula of tubercle bacilli are highly susceptible to concentrations of penicillin as low as 1 unit per cc in the Tween-albumin medium. In the oleic acid-albumin medium, penicillin in a concentration of 100 units per cc causes no inhibition of growth, even with the smallest inocula.

Preliminary experiments indicate that in both the solid and liquid oleic acid-albumin medium, penicillin in concentrations of 50 to 100 units per cc may prove a valuable adjunct in culturing tubercle bacilli from contaminated materials.

[13] McCulloch, E. C., *Disinfection and Sterilization*, 2nd Edition, Lea and Febiger, Phila., 1945.

[14] Fisher, M. W., *Am. Rev. Tuberc.*, in press.

[15] Corper, H. J., and Cohn, M. L., *Am. Rev. Tuberc.*, 1946, **53**, 575.

16005

Some Antibacterial Properties of Mandelamine (Methenamine Mandelate).

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In recent years, the sulfonamide drugs and the antibiotics have dominated the field of chemotherapy. The successful use of these drugs in the treatment of urinary tract infections, however, is dependent upon an unobstructed urine flow plus adequate kidney function. Further, administration of these drugs requires close medical supervision and, finally, invading organisms tend to develop resistance to both classes of drugs. For these reasons, a relatively non-toxic drug which can be administered by mouth over protracted periods of time to ambulant patients would be highly desirable. According to Carroll and Allen[1]

[1] Carroll, G., and Allen, H. N., *J. Urology*, 1946, **55**, 674.

TABLE 1.
Stock Laboratory Strains in Normal Sterile Urine Buffered at pH 5.5. Minimal bacteriostatic and bactericidal concentrations of mandelamine, streptomycin, and sulfathiazole given in mg %. Each figure is the average of 5 different series of tests.

	Mandelamine		Streptomycin		Sulfathiazole	
	Static	Cidal	Static	Cidal	Static	Cidal
E. coli 64	50	50	20	20	5	10
" " 4R5190	50	50	20	20	7.5	20
A. aerogenes 129	25	25	20	20	3	5
K. friedlanderi type AF12	12.5	12.5	5	5	5	10
" " " BEGS	25	25	5	5	5	10
St. aureus 209	15	25	10	20	2.5	10
" " SM	20	25	40	40	10	10
Ps. aeruginosa ATCC 9029	40	40	40	40	6.25	20

and Kirwin and Bridges,[2] mandelamine appears to be such a drug. In order to obtain an additional evaluation of the drug, the activities of mandelamine, sulfathiazole and streptomycin against organisms commonly found in urinary tract infections were compared, and the development of resistance by representative organisms to all three drugs was studied. The findings are set forth in the following paragraphs.

Experimental. The medium used throughout this work consisted of normal male urine adjusted to pH 5.5 or 6.5 with phthalate and phosphate buffers in .03 to .06 molar concentrations, respectively. The pH was determined before and after incubation by means of a Coleman model 3 pH electrometer. In the presence of effective bacteriostasis, the pH did not vary by more than 0.5 pH units in any experiment. The inoculum was always 0.1 ml of a 1:5000 dilution of an 18-hr. broth culture. Suitable control tubes of buffered and unbuffered nutrient broth, and of buffered urine were included in each test. If any of the control tubes showed no growth, the whole series was discarded. Eight stock laboratory strains of bacteria commonly found in urinary tract infections were studied at pH 5.5 and 9 strains of organisms recently isolated from urinary tract infections were studied both at pH 5.5 and 6.5. Minimal bacteriostatic concentrations of mandelamine, sulfathiazole and streptomycin were determined by means of a serial 2-fold dilution method. End-points were taken as the lowest drug concentrations at which no growth occurred within 72 hours. Bactericidal activity was determined by subculturing 0.25 ml from each apparently negative tube into 5 ml of nutrient broth followed by examination for visible growth after 72 hours.

Attempts were made to induce resistance in 6 different organisms to streptomycin, sulfathiazole and mandelamine as follows: 0.5 ml of an 18-hour nutrient broth culture was used to inoculate 5 ml of sterile urine, at pH 5.5, in the presence of serial 2-fold dilutions of the individual drugs. After 72 hours incubation, 0.5 ml samples were taken from the tubes containing the highest drug concentrations in which growth had occurred. These were transferred into tubes containing a higher series of drug concentrations, and so on. Finally, when organisms had developed resistance to streptomycin or to sulfathiazole, the antibacterial activity of mandelamine against the resistant organisms was determined.

Results and Discussion. The average minimal bacteriostatic and bactericidal concentrations of sulfathiazole, streptomycin and mandelamine at pH 5.5 against the 8 stock laboratory strains are shown in Table I. The results indicate that minor differences in potency exist but, in general, the activities of all 3 drugs are of the same order of magnitude.

The data obtained at pH 5.5 and 6.5 with the 9 strains recently isolated from urinary tract infections are shown in Table II. The results indicate that streptomycin is somewhat less active at pH 5.5 than at 6.5, the difference being related, presumably, to destruction of

2 Kirwin, T. J., and Bridges, J. P., *Am. J. Surg.*, 1941, **52**, 477.

† We are indebted to Miss Anna M. Kelly and Miss Mary V. Rothlauf for technical assistance.

TABLE II.

Recently Isolated Strains in Normal Sterile Urine Buffered at pH 5.5 and 6.5. Minimal bacteriostatic and bactericidal concentrations in mg %. Each figure is the average of 5 different series of tests.

pH	Mandelamine				Streptomycin				Sulfathiazole			
	5.5		6.5		5.5		6.5		5.5		6.5	
	static	cidal	static	cidal	static	cidal	static	cidal	static	cidal	static	cidal
E. coli Grogan	25	25	62.5	62.5	10	15	5	10	1.56	5	1.25	20
" " Fernande	50	50	150	150	15	15	5	10	10	20	0.6	0.6
" " Travis	50	50	125	125	20	20	5	5	3	10	1.25	>40
A. aerogenes Harrington	30	30	62.5	62.5	3	5	2.5	2.5	20	20	2.5	40
St. albus No. 6	25	50	75	75	20	20	10	15	.625	2.5	1.25	20
" " Agostini	125	—	75	75	40	—	10	20	5	—	1.25	5
St. hem. No. 9	62.5	—	50	50	20	—	20	20	2.5	—	.6	.6
Ps pyocyaneous No. 7	125	125	200	200	40	40	20	30	5	40	20	40
Ps. aeruginosa Fox	62.5	62.5	200	200	20	20	5	10	2.5	10	2.5	20

TABLE III.

Induced Resistance to Sulfathiazole, Streptomycin, and Mandelamine. Minimal bacteriostatic concentrations of mandelamine before and after development of resistance to sulfathiazole and to streptomycin. Concentrations given in mg %. Each figure is the average of 2 different series of tests.

Organism	Sulfathiazole			Streptomycin			Mandelamine					
	E. coli G	*Aero aero* H	*S. aur.* 209	*E. coli* 64	*Aero aero* 129	*S. aur.* SM	*E. coli* G	*Aero aero* H	*S. aur.* 209	*E. coli* 64	*Aero aero* 129	*S. aur.* SM
Original min. bacteriostatic conc.	20	20	20	50	50	15	25	100	200	200	200	20
	(*ca.* 75 mg %)											
Final min. bacteriostatic conc.	>Sat.	>Sat.	>Sat.	1500	1200	1500	75	300	75	600	150	20
Increase in resistance	>4X	>4X	>4X	30X	24X	100X	3X	3X	—1.7X	3X	—	—
Number of transfers	5	3	3	8	10	9	9	10	10	9	10	9
Original min. bacteriostatic conc. of mandelamine	25	100	200	200	200	20						
Min. bacteriostatic conc. of mandelamine after induction of resistance	75	300	500	150	75	37.5						

the drug at the lower pH. Sulfathiazole is approximately equally active at both pH levels, but the threat of sulfonamide urolithiasis[3] at the lower level should preclude its use in acidic urine. Mandelamine is more effective at pH 5.5 than at 6.5 but at either pH bactericidal concentrations are low enough to permit ready attainment *in vivo* by appropriate dosing.[1] In connection with this heightened activity at the lower pH values, it is to be noted that mandelamine is an acidifying agent, and Carroll and Allen[1] report, "The urine of all patients (200) in the series but 8 became or remained acid on the therapeutic regimen without other medication or restriction of diet or fluid intake."

As shown in Table III, organisms grew in urine which was saturated, at pH 5.5, with sulfathiazole. The degree of resistance which can be developed is necessarily limited by this restricted solubility of the drug, but within this limitation, a 4-fold increase in resistance occurred after as little as 3 to 5 transfers. Resistance to streptomycin developed more rapidly, an increase of 24- to 100-fold being found after 8 to 10 transfers. In contrast to the foregoing, resistance to mandelamine either did not appear, or if it did, it appeared slowly and only to a limited degree; for example, after 9 to 10 transfers there was no change in the susceptibility of 3 organisms, while with the remaining organisms there was only a 3-fold increase in resistance. From the same table, it appears that sulfathiazole-resistant organisms displayed a slightly increased resistance to mandelamine, while the streptomycin-resistant bacteria did not. Further, it appears that mandelamine retained its bactericidal activity against *A. aerogenes H* and *S. aureus* 209, but was only bacteriostatic to the other 4 organisms. These preliminary findings, which suggest that resistance developed against one drug may alter susceptibility to another chemically unrelated drug, are somewhat anomalous, and are being investigated further.

Summary. A comparison of mandelamine, streptomycin and sulfathiazole against 8 stock laboratory strains and 9 strains of organisms recently isolated from urinary tract infections indicates that their activities are not widely different.

In contrast to the results obtained with streptomycin and sulfathiazole, resistance to mandelamine either did not appear, or if it did, it appeared slowly and to a questionable degree in 3 organisms, while in 3 other organisms, resistance did not appear at all.

Organisms rendered resistant to sulfathiazole or streptomycin remain susceptible to mandelamine.

[3] Scudi, J. V., *Am. J. Med. Sciences*, 1946, **211**, 615.

16006 P

Differences During Dicoumarol Therapy in the Quick and Russell Viper Venom Methods for Prothrombin Determination.*

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We recently observed a patient with thrombophlebitis and pulmonary emboli who died in a state of hemorrhagic diathesis during dicoumarol therapy. An average daily dose of 240 mg of dicoumarol administered for 14 days had failed to decrease the prothrombin to the desired therapeutic level as measured by the Russell viper venom modification[1,2] of Quick's method.[3] Subsequently the methods

* This study was aided by a grant from the National Research Council.

[1] Fullerton, H. W., *Lancet*, 1940, **2**, 195.

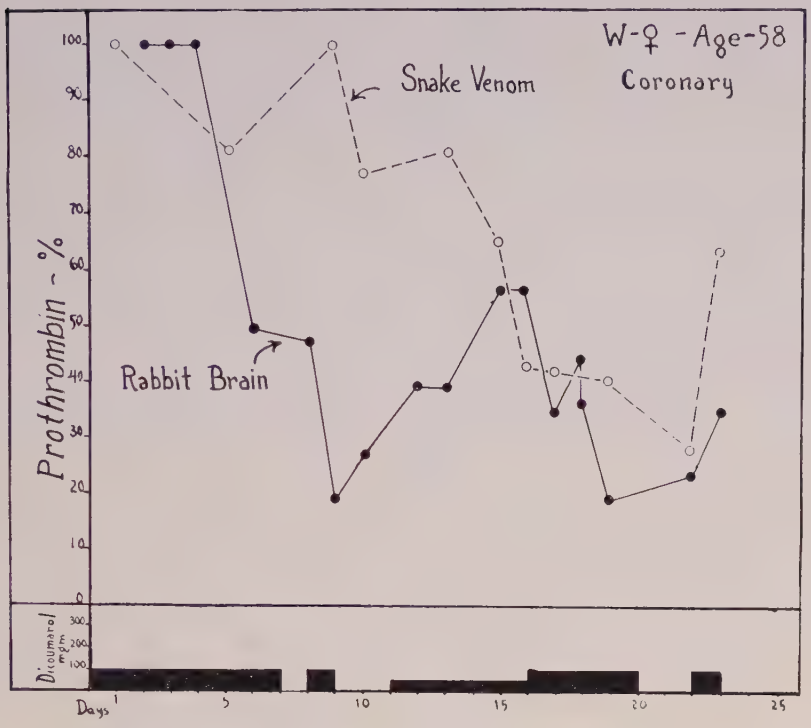

FIG. 1.

Variations in prothrombin during dicoumarol therapy as measured by Quick's method (rabbit brain thromboplastin) and the Russell viper venom modification. Quick's method can be correlated with changes in dicoumarol dosage, the venom method cannot.

of Quick[3] and Page[2] were compared in seven patients receiving dicoumarol therapy.

Striking differences were observed in the quantitative levels of prothrombin as measured by the two methods. Fig. 1 illustrates graphically these variations in a white female who was given dicoumarol orally for coronary occlusion. The therapeutic amounts of dicoumarol, including the initial dosage and changes in the amount of the drug, are closely correlated with the prothrombin levels as determined by the method of Quick, using rabbit brain as the thromboplastin. There is a slow gradual decrease in the prothrombin level as measured by the Russell viper method which cannot be correlated with the Dicoumarol therapy.

Other patients exhibited the same differences. One developed hematuria and it was observed that the prothrombin was less than 5% of normal by the method of Quick and 61% of normal by the Russell viper venom technique. The results of the two methods were not comparable until 4 days later. In all of the patients observed the prothrombin as determined by the method of Quick decreased to the desired therapeutic levels long before there was a decrease by the snake venom method. The levels of prothrombin as determined by the method of Quick could be correlated with the clinical hemorrhagic state of the patients, whereas the amount of prothrombin as determined by the Russell viper venom method resulted in false safe levels. Frequently the patients received too much dicoumarol because of the results of the venom method.

Conclusions. The quantitative values of prothrombin as determined by the method of Quick using rabbit brain thromboplastin can be correlated with dicoumarol therapy and

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[3] Quick, A. J., *J. A. M. A.*, 1938, **110**, 1658.

clinical hemorrhagic tendencies. The levels of prothrombin as determined by the Russell viper venom modification of Quick's method cannot at all times be correlated with the clinical state of the patient and the dicoumarol therapy. The control of dicoumarol therapy by the Russell viper venom method for prothrombin determination is a dangerous procedure. The results may be interpreted as being in a safe therapeutic level of prothrombin when actually the patient may be in a critical potential or actual hemorrhagic condition, this state being determined both by the Quick method and clinical observations.

16007

Electrocardiogram-Electroencephalogram Relations in Cardiac Arrhythmias.

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The initiating relationship of the brain, and in particular of the hypothalamus, to cardiac arrhythmias has been established on both experimental[1-4] and clinical[5-10] grounds. Although a proportion and probably a majority[11,12] of the arrhythmias may find their prime origin in intracardiac derangements, numerous cases of various cardiac arrhythmias are on record[13,14,15] in which the heart is normal. This study contains electroencephalographic data in cases of arrhythmic patients.

The electroencephalograms (EEG) were taken using the hypothalamic lead. Confirmatory electrocardiograms (EKG) were taken in each instance, except one, prior to the EEG. The EEG classification of Gibbs was used in determination of normalcy.† The results are summarized in the table, and tracings in Case 1-1a are reproduced in Fig. 1.

Interpretation. These cases may be divided

* Published with permission of the Chief Medical Director, Department of Medicine and Surgery, Veteran's Administration, who assumes no responsibility for opinions expressed or conclusions drawn by the author.

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† The writer wishes to acknowledge the electroencephalographic interpretations kindly furnished by David R. Talbot.

TABLE I.

Patient No.	Sex	Age	Other diagnosis	Arrhythmia by EKG	Preliminary EEG	Medication for conversion to normal rhythm	EEG with normal rhythm	EKG with normal rhythm
1.	♂	32	None	Paroxysmal auricular fibrillation-flutter	Abnormal Much fast 12-20 C/S	Quinidine	Normal	Normal
1a.	♂	32	None (Readmission)	Same	Same	,,	,,	,,
2.	♂	50	None	Paroxysmal auricular flutter	,,	,,	,,	,,
3.	♂	50	,,	Paroxysmal auricular fibrillation-flutter	None taken	,,	,,	,,
4.	♀	58	,,	History of parox. tachycardia, 30 years	—	—	Abnormal 4-9 C/S, High ampl. Alpha	Questionable evidence of myocardial damage
5.	♂	50	,,	History of parox. auric. fibrillation-flutter, 12 years	—	—	Borderline, Mixed frequency 8-22 C/S	Normal
6.	♂	50	Arteriosclerosis; cardiac hypertrophy	Auricular fibrillation	Normal	None attempted	—	—
7.	♂	63	Buerger's disease; amputations of 4 extremities	Extrasystoles, multiple foci. (Evidence of old myocardial infarction.)	,,	,, ,,	—	—
8.	♂	55	History of rheumatic fever	Auricular fibrillation	,,	Quinidine	Normal	—

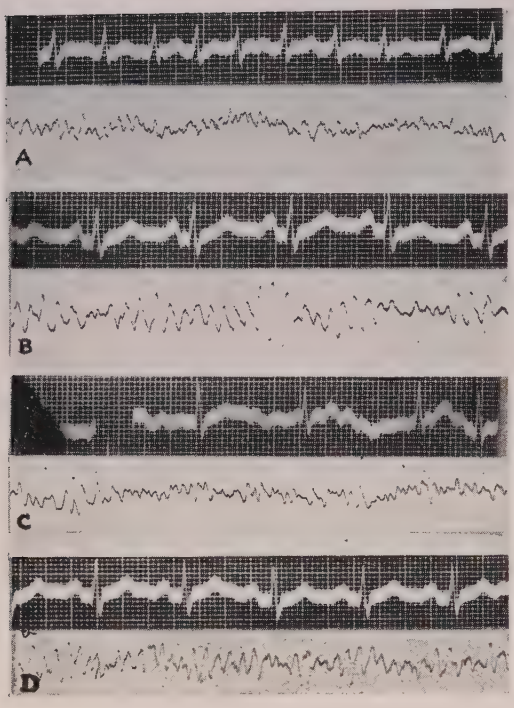

FIG. 1.
Electroencephalograms and electrocardiograms in a case of paroxysmal auricular fibrillation-flutter.
A. 3-20-47. C. 6-20-47 (Readmission).
B. and D. After conversion to normal cardiac and encephalic rhythm, using quinidine.
B. 5-8-47. D. 7-16-47.
EKG—Lead II. EEG—Left.

into 2 general groups: Those in which there is EKG history and physical evidence of myocardial damage, and those without such evidence. Cases 1, 2, 3, 4, and 5 fall within the second category. Reverse cerebral excitation is not supported by findings in cases 6, 7 and 8. EEG abnormality in a case with a history of long-standing paroxysmal arrhythmia without definite evidence of myocardial damage (Cases 4, 5) may or may not be pertinent to the cardiac arrhythmia. Experimental[4] and clinical[13] demonstration of the variety of possible neurogenic cardiac arrhythmias does not make feasible any separation at this time on the basis of type of arrhythmia. Factors not associated with the arrhythmia may cause an abnormal EEG and therefore the indication that an abnormal EEG may accompany a central excitation causing the cardiac arrhythmia is gained only from Cases 1-1a, and 2. Even here it will be necessary to investigate the possibility of cerebral circulatory insufficiency secondary to the cardiac arrhythmia.

Summary. Evidence is presented to indicate that electroencephalographic abnormality coincident with neurogenic cardiac arrhythmia may disappear with restoration of normal cardiac rhythm.

16008 P

Pressure Stimulation of Peripheral Nerves.

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Clinical experience suggests that pressure upon peripheral nerves or nerve roots is accompanied by pain, as well as by alterations in sensation and other neurological changes. The radicular pains caused by neoplasms of the spinal canal, the root pains arising from herniations of the intervertebral discs and the pain caused by pressure on the brachial plexus of cervical ribs or thickened scalenus muscles are common examples. Pain occurs early and persists throughout the course of these conditions and has been attributed to the stimulating effect of the steady pressure against the nerve.

On the other hand, experimental physiological studies have repeatedly demonstrated that nerve fibers are relatively inexcitable to steady or even slowly changing stimuli. The classical studies on electrical stimulation of

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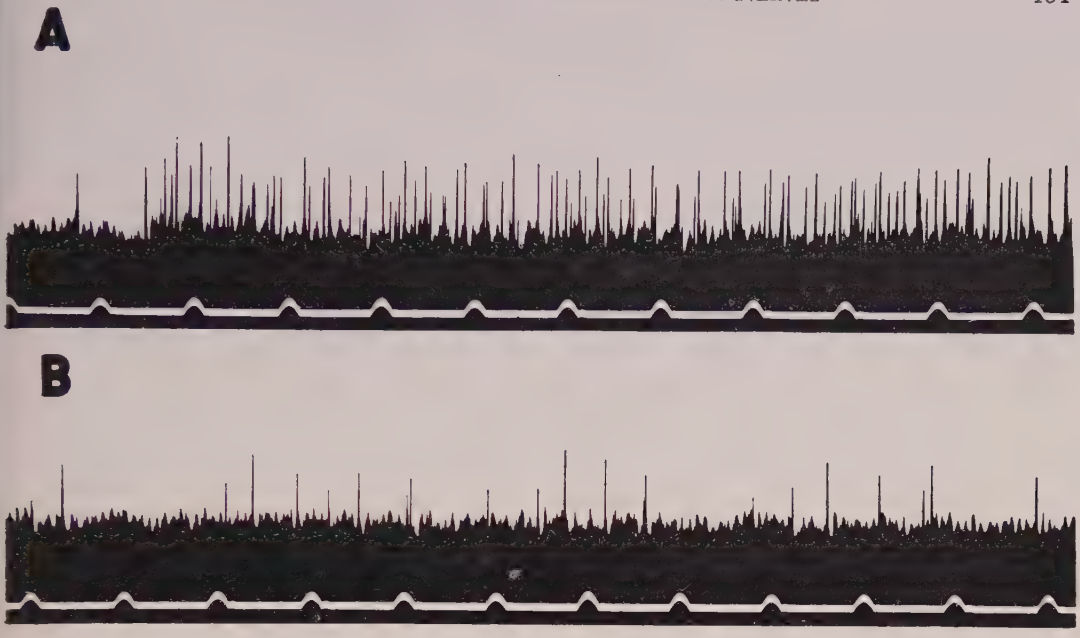

FIG. 1.

Impulses in the sciatic nerve of a frog, initiated by compression of a 5 mm length of nerve with a 40 g weight. Reoord A shows the impulses discharged when the weight was first suddenly applied, record B after the same weight had been kept on the nerve continuously for 10 minutes. Timer indicates 1/5 seconds.

A few larger and briefer impulses appeared during the first 1/5 second after application of the weight, as described in the text, but their oscillograph tracings were too faint for photographic reproduction.

nerve emphasize that stimulation ordinarily occurs only on the make and break of a circuit, whereas constant currents do not stimulate at all. It is obvious, therefore, that there is a discrepancy between the clinical and experimental views. This may mean that the effects observed clinically are more than the effects of simple pressure stimulation or that the classical notions of stimulation have tended to minimize certain properties of nerve which might allow for repetitive response to steady, unchanging stimuli. Recent experiments have indeed shown that under suitable conditions constant electric currents may evoke a repetitive response (Erlanger and Blair,[1] and Rosenblueth[2]). Moreover, very long sustained activity can be caused by steady chemical stimulation (Brink and Bronk,[3] Lehman[4]).

The experiments described in the present paper were undertaken to determine whether steady pressures applied to an isolated nerve preparation can similarly give rise to a continuous series of nerve impulses.

Pressure transients, that is, sudden, non-injurious increases or decreases in pressure, have been shown to initiate impulses in the sciatic nerve of the frog (Blair,[5] Schmitz and Schaefer[6]) whereas steady pressure of sufficient intensity will block conduction (Gasser[7]). It is known also that, if the nerve is subjected to an increased hydro-static pressure throughout its entire length, its excitability will be modified (Grundfest[8]). To the writers' knowledge, however, there has been no laboratory demonstration that the application of

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steady pressure to a localized portion of a peripheral nerve will produce a steady discharge of impulses. The purpose of this paper is to report such observations on the sciatic nerve of the frog by means of an amplifier and oscillographic recording.

Methods. The sciatic nerve was removed from the leg of a frog and placed in a moist chamber so that the large proximal portion of the nerve rested on a waxed ebonite platform. A second ebonite block fitted over this platform and, when in position, pressed directly on a 5 mm length of the nerve. Pressures were graded by loading the block with various weights. Nerve impulses initiated by this pressure were recorded as action potentials in the smaller distal branches of the nerve.

Results. When weights of from 30-50 g were dropped on the nerve from a height of 2 mm, two types of response were elicited. At the moment of contact a transitory burst of fast, large action potentials was recorded. At the same time there was a discharge of slower and smaller impulses, which continued as long as the pressure was applied (Fig. 1). During this period the asynchronous discharge of the nerve showed a diminution in frequency analogous to "adaptation". Some fibres would "adapt" completely after a few minutes, *i.e.*, cease to respond, whereas others continued to discharge for as long as 15 minutes. Observations were terminated at the end of this period, at which time the activity had reached a fairly constant level. Gradual application of the weight elicited a discharge of the slower impulses only. When the response had attained a fairly constant level some minutes after application of the stimulus, further increase of pressure increased the frequency of discharge and occasionally brought in additional fibers. Withdrawal of the weight was followed by a gradual cessation of the response.

In another series of experiments the sciatic nerve was freed by dissection in the thigh region of the frog's leg, leaving the nerve in functional continuity with the spinal cord. The spinal canal was opened to expose the sensory and motor roots which were then cut close to the cord and placed in turn on the recording electrodes. The dissected peripheral end of the nerve was arranged for pressure stimulation as in the above experiments. With this arrangement it was possible to determine whether the impulses initiated by pressure were traveling in motor or sensory fibers. A repetition of the foregoing procedures showed that the prolonged response to pressure occurred only in the dorsal roots, *i.e.* sensory fibers. The sole response from the motor roots was the transitory burst of fast impulses when the stimulus was applied. The fact that later activity was restricted to sensory fibers may depend on the presence of small as well as large fibers in this category, while the motor nerves consist almost exclusively of large diameter fibers. The prolonged phase of the pressure response occurs in fibers of small diameter as indicated by the relatively small amplitude and relatively long duration of the individual action potentials.

16009

Persistence of Streptomycin Resistance During Subcultures in Streptomycin Free Media.*

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Previous studies on the persistence of streptomycin resistance in bacteria have yielded variable results.[1-7] These studies, most of which were done with resistant variants that appeared following the growth of the organisms in culture media containing streptomycin, have indicated that the resistance of organisms usually remains essentially unchanged after prolonged storage in, or repeated transfers through streptomycin-free media.[1,6,7] In many instances, however, a diminution in the degree of resistance was demonstrated after varying numbers of transfers.[2-5] On the other hand, an apparent increase in resistance has also been observed in occasional strains.[5] Since, in all probability, these studies were concerned with bacterial populations of varying sensitivity or resistance, the results observed necessarily reflected only the most resistant strains which could multiply in the streptomycin-containing media used in the tests for sensitivity.

The purpose of the present study was to examine a group of resistant strains of various organisms, some obtained directly from patients under treatment with streptomycin and others derived *in vitro* after growth in streptomycin containing media, with a view to determining whether any loss of resistance took place in the course of 100 serial transfers through streptomycin free broth. In this study due cognizance was taken of the possibility that the final cultures might contain mixtures of organisms of varying sensitivity or resistance.

Experimental. The 13 strains selected for this study and the sources of these strains are listed in Table I. All were highly resistant to streptomycin as determined by a serial dilution method in broth.[8] Two of them (Nos. 6 and 10) were inhibited by 50,000 μg/ml but not by 10,000 μg/ml and the others were not inhibited by 50,000 μg/ml, the highest concentration used in these studies. Five of the strains (Nos. 1, 4, 7, 9 and 12) had been isolated several months earlier from patients under treatment with streptomycin; 7 (Nos. 2, 5, 6, 8, 10, 11, and 13) were resistant strains derived from originally sensitive organisms by successive passage through media containing increasing concentrations of streptomycin[7] and strain No. 3 was isolated only a few days before the present study began and was derived from the same parent strain as No. 2 during the course of a single 24-hour exposure to streptomycin. All of the strains except the latter one had been stored on heart infusion agar slants for 4 months at 40°C.

Each of the strains was checked for purity by first streaking on agar plates and selecting single colonies which were then transferred to brain heart infusion broth (Difco) at pH 7.4 and tested for streptomycin sensitivity. These broth cultures represented the first of the projected 100 transfers. Subsequent transfers

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TABLE I.
Streptomycin Sensitivities of Resistant Strains After Varying Numbers of Subcultures in Broth.

Strain				Number of transfers				
No.	Organism	Patient	Source*	1	26	57	75	100
1	*A. aerogenes*	E.F.	a	>50†	>50	>50	>50	>50
2	,,	,,	b	,,	,,	,,	,,	,,
3	,,	,,	c	,,	,,	,,	,,	,,
4	,,	A.P.	a	,,	,,	,,	,,	,,
5	,,	,,	b	,,	,,	,,	,,	,,
6	*K. pneumoniæ*	C.M.	b	50	,,	,,	,,	,,
7	,, (atypical)	C.H.	a	>50	,,	,,	,,	,,
8	,, ,,	,,	b	,,	,,	,,	,,	,,
9	*E. coli*	M.K.	a	,,	,,	,,	,,	,,
10	,,	,,	b	50	50	,,	50	50
11	*Ps. aeruginosa*	C.M	b	>50	>50	,,	5	1
12	Paracolon bacillus	W.T.	a	,,	,,	,,	>50	>50
13	,, ,,	,,	b	,,	,,	,,	,,	,,

* a = Isolated from patients during streptomycin treatment.[8]
b = Derived from sensitive strain after repeated subculture in increasing concentrations of streptomycin.[7]
c = Derived from same sensitive strain as No. 2 after exposure to streptomycin for 24 hours.
† Concentration of streptomycin, mg/ml, required for complete inhibition of growth in brain heart infusion broth, pH 7.4, with an inoculum of 10^{-4} ml.

were then made from the broth cultures by means of a 2 mm platinum loop. The culture tubes each contained approximately 5 ml of the brain heart infusion broth and were incubated for a period of 24 hours prior to use. The transfers were made at daily intervals at first but, as the organisms became adapted to growth on the medium, it was possible to make the transfers more frequently and as many as 3 could be made in the course of 24 hours after the 70th transfer. The cultures were examined at different stages of the experiment by streaking on heart infusion agar and on eosin-methylene blue plates in order to detect possible contaminants. All cultures remained free of contaminating organisms during the course of the experiment.

The 1st, 26th, 57th and 100th transfers were tested for streptomycin resistance by the serial dilution method in broth and the results are shown in Table I. Sub-cultures were also made at these times on agar slants and stored at 4°C for reference. All but 1 of the 13 cultures tested appeared to retain their initial streptomycin resistance through 100 transfers in streptomycin free broth. No. 11, a strain of *Pseudomonas aeruginosa* initially resistant to over 50,000 μg/ml was inhibited by 5000 μg/ml after 75 transfers and by 1000 μg/ml after 100 transfers.

Although 12 of the strains had apparently remained resistant throughout the experiment, it was not possible to say that there were not, in fact, some susceptible cells present which were masked by the more resistant ones under the conditions of the sensitivity test. If such were the case, the occurrence of the more susceptible cells might be detected by doing bacterial counts of a series of agar plates containing graded concentrations of streptomycin and seeded with the same number of organisms. This was attempted with each of the organisms using the broth culture of the 100th transfer and streptomycin concentrations of 10,000, 1000, 100, and 10 μg/ml. Additional plates containing intermediate concentrations of 316. and 31.6 μg/ml were included in the case of strains 10 and 11 which, in preliminary trials, showed marked changes.

The plates were prepared by introducing first 1.0 ml amounts of streptomycin solutions containing 10 times the desired final concentration, then 0.1 ml of a 10^{-6} dilution of the cultures, which experience had shown would yield about 200-300 colonies in the absence of streptomycin, and, finally, 8.9 ml of melted agar. Care was taken to avoid mixing the organisms with the streptomycin before the agar was added. Control plates were poured with the same inoculum of organisms and no strep-

TABLE II.
Number of Colonies Grown from 0.1 ml of a 10^{-6} Dilution of the 100th Broth Transfers Seeded in Agar Containing Graded Concentrations of Streptomycin.

Strain	Final concentration of streptomycin, μg/ml						
	10,000	1,000	316	100	31.6	10	0
1*	200	202		187		194	199
2	560	585		552		586	592
3	345	340		390		390	357
4	352	347		368		442	370
5	225	270		219		240	234
6	470	480		478		500	476
7	245	246		267		280	250
8	290	289		305		280	300
9	215	228		209		247	241
10*	0	0	0	47	138	139	120
11*	0	0	0	0	98	170	179
12	290	300		300		260	323
13	280	285		260		304	290

* Average count of 4 plates for each of these strains.

tomycin. Colony counts were made after incubation for 48 hours.

The results are shown in Table II. Striking differences in the counts were noted in 2 cultures: No. 11, the strain of *Ps. aeruginosa* that was already noted to have become reduced in resistance by the serial dilution method in broth and No. 10, a strain of *E. coli*. These 2 strains produced no colonies in concentrations of streptomycin greater than 31.6 and 100 μg/ml, respectively. The remaining 11 strains showed no significant difference between the counts obtained in the streptomycin-free plates and in the plates containing streptomycin in concentrations up to 10,000 μg/ml.

It was now of some interest to determine, if possible, when in the course of the 100 transfers the more sensitive variants made their appearance and became detectable in appreciable numbers. It was also desirable to determine whether larger inocula would bring out a greater heterogeneity within the cultures of the 2 strains that had apparently become more sensitive. For these purposes, the cultures of strains 10 and 11 that had been stored on agar after the 1st, 26th, 57th and 75th transfer were subcultures in broth so that they now represented the 3d, 28th, 59th and 79th transfer, respectively. The previous experiment was now repeated with each of these subcultures, using the same inoculum, namely, 0.1 ml of a 10^{-6} dilution. In addition, the same procedure was carried out at the same time with an inoculum of 0.1 ml of the same cultures undiluted—that is, a million times as many organisms were seeded in the same manner.

The results are shown in Table III. In the case of Strain 11 there was a sufficient difference in the number of colonies that developed from both the large and the small inoculum to indicate that an appreciable number of more sensitive cells had already appeared after 28 transfers, while there can be no doubt that this was the case after 59 transfers. This appears more clearly from the results obtained with the smaller inoculum. With Strain 10 there was similar evidence of the presence of cells of decreased resistance (increased sensitivity) at the 59th transfer.

Discussion. Of the 13 cultures studied, 11 showed no diminuition in streptomycin resistance, by the methods used, during the course of 100 transfers in streptomycin free broth. In the case of 2 strains, however, the evidence indicates that organisms appeared after serial subcultures which were more sensitive to streptomycin than those of the starting cultures. The strain of *Ps. aeruginosa,* No. 11, which had originally become resistant *in vitro,* showed no growth in a concentration of 1000 μg of streptomycin per ml when tested after 100 transfers by the serial dilution method in broth. However, when a smaller inoculum was seeded in plates of agar containing graded concentrations of streptomycin (in this in-

TABLE III.

Numbers of Colonies Grown in Agar Containing Graded Concentrations of Streptomycin and Seeded with 0.1 ml Amounts of Undiluted and of 10^{-6} Dilution of Cultures of Resistant Organisms After Varying Numbers of Transfers in Streptomycin-free Broth.

Strain	No. of transfers	Inoculum: 0.1 ml of undiluted culture — Conc. of streptomycin, μg per ml							Inoculum: 0.1 ml of 10^{-6} dilution of culture					
		10,000	3,160	1,000	316	100	31.6	10	10,000	1,000	316	100	31.6	10	0
10	3	++++*	++++	++++	++++	++++	++++	++++	50	62	55	63	70	75	63
	28	+++	++++	++++	++++	++++	++++	++++	89	105	100	88	104	92	100
	59	+	++++	++++	++++	++++	++++	++++	0	90	85	77	83	83	81
	77		3	40	64	4,260	++++	++++	0	0	0	38	130	129	137
	100		32	45	758	3,000	++	++++	0	0	0	47	138	139	120
11	3		+++	+++	+++	++++	++++	++++	200	230	238	220	235	218	226
	28		590	600	800	850	860	++++	30	230	400	754	680	700	740
	59		154	211	260	267	690	++++	0	0	0	5	150	300	342
	77		48	67	57	68	102	++++	0	0	0	0	103	200	226
	100		34	54	65	300	332	++++	0	0	0	0	98	170	179

* +, ++, +++ indicate increasing numbers of colonies beyond the number that could be counted.

stance about 179 organisms per 10 ml of agar) 98 colonies developed in the plate containing streptomycin in a concentration of 31.6 μg/ml and none grew in the plates containing 100 μg or more per ml. On the other hand, when a much larger inoculum was used, namely 0.1 ml of the undiluted culture containing 179 million organisms, 32 colonies were counted in the plate containing 10,000 μg of streptomycin per ml and larger numbers of colonies developed in the plates which contained lower concentrations of the antibiotic.

The strain of *E. coli,* No. 10, was originally not quite as resistant as the other strains in that it was completely inhibited by 50,000 μg/ml. The serial dilution method in broth did not demonstrate any diminution in resistance of this strain during 100 transfers but the agar pour plate method, utilizing a small inoculum and serial concentrations of streptomycin, gave a picture similar to that found with Strain 11.

In interpreting the sensitivity tests, it should be borne in mind that the inoculum used in the serial dilution method in broth, as employed here, contains 0.5 ml of a 10^{-4} dilution of culture, or something in the order of 100,000 organisms. The sensitivity indicated by this method will correspond to that of the most resistant cells in the inoculum, or possibly to that of the most resistant variants that emerge during the course of the test, irrespective of their numbers, provided only that they remain viable and are capable of good growth in the media within the period of observation. If the number of resistant organisms be less than 1 per 100,000 cells, their presence might well go undetected unless a sufficiently large number of tubes were used.

Both Strain 10 and Strain 11, had originally become resistant *in vitro.* No loss of resistance was noted in any of the resistant strains which had been isolated directly from patients. The total number of strains studied, however, is too small to permit any comparison of resistant strains with respect to their origin.

Summary. Each of 13 strains of gram-negative bacilli that were highly resistant to streptomycin was passed through 100 serial

subcultures in broth. Evidence was obtained which indicated that appreciable numbers of streptomycin sensitive cells appeared in 2 of these strains: in one instance after 28 subcultures and in the other after 59 transfers. The 11 remaining strains appeared to retain their resistance.

16010

Circulating Antibodies in Vitamin-Deficiency States: I. Pyridoxin, Riboflavin, and Pantothenic Acid Deficiencies.*

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In the study of nutrition any measure of animal performance can be utilized for the evaluation of diets. Antibody production, the normal physiological response to an antigenic stimulus, might be expected to provide a measure of the nutritional status of an experimental animal. Inasmuch as relatively few conclusive studies of this type are reported in the literature, the ability to form antibodies seems a worthy supplement to other measures of the role of various dietary factors.

Few studies have appeared concerning the effect of specific vitamin B deficiencies upon antibody production in the rat. Ruchman[1] reported that neither riboflavin nor thiamin deficiencies affected antibody production. More recently, Stoerk and Eisen[2] found reduced serum antibody concentrations in pyridoxin deficiency. Stoerk, Eisen, and John[3] have reported normal serum antibody concentration in thiamin, riboflavin, and pantothenic acid-deficient rats. Washed sheep erythrocytes were employed as the antigen in these experiments.

The present report presents data concerning the effect of pyridoxin, pantothenic acid, and riboflavin deficiencies upon antibody production by the rat in response to human red blood cells as antigenic stimulus.

Experimental. Two series of experiments differing only in the immunization procedure were conducted. Male weanling albino rats of the Sprague-Dawley strain were distributed into groups of litter mates as indicated in Table I. The animals were housed individually in wide-mesh screen-bottom cages and weighed weekly. With the exception of the group which received a colony diet (Arcady Farms) *ad libitum*, the animals were placed on a dietary regimen which consisted of a basal diet and additional vitamins in the form of a daily pill. The basal diet had the following percentage composition: sucrose, 56.76; Labco "vitamin-free" casein, 25.00; salts,[4] 4.00; cod liver oil, 2.00; hydrogenated vegetable oil, 10.00; corn oil, 2.00; choline chloride, 0.20; *p*-aminobenzoic acid, 0.01; *i*-inositol, 0.03; and 2-methyl-1, 4-naphthoquinone, 0.001. Each of the pills given to the two control groups supplied the following vitamins: thiamin, 40 γ; riboflavin, 60 γ; calcium pantothenate, 200 γ; pyridoxin, 50 γ; biotin, 1 γ; folic acid, 1 γ; and nicotinic acid, 100 γ. For the pyridoxin, riboflavin, and pantothenic acid-deficient groups, the appropriate vitamin was omitted from the

* Supported in part by a grant from Swift and Company, Chicago, Ill.

1 Ruchman, I., *J. Immunol.*, 1946, **53**, 51.

2 Stoerk, H. C., and Eisen, H. N., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 88.

3 Stoerk, H. C., Eisen, H. N., and John, H. M., *J. Exp. Med.*, 1947, **85**, 365.

4 Jones, J. H., and Foster, C., *J. Nutrition*, 1942, **24**, 245.

TABLE I.
Summary of Growth and Food Consumption Records.

Group	No. of rats	Body wt*		Daily food consumption‡
		Initial	Final†	
	Series I.			
Pyridoxin-deficient	5	39	129	5.7
Riboflavin-deficient	5	39	48	
Pantothenic acid-deficient	5	41	100	
Inanition control	5	39	157	5.7
Control—*ad libitum*	5	40	329	
Colony diet	5	39	286	
	Series II.			
Pyridoxin-deficient	5	46	134	5.1
Riboflavin-deficient	6	42	59	3.2
Pantothenic acid-deficient	2	45	91	4.0
Inanition control	5	45	161	5.1
Control—*ad libitum*	5	43	348	11.9
Colony diet	5	43	289	

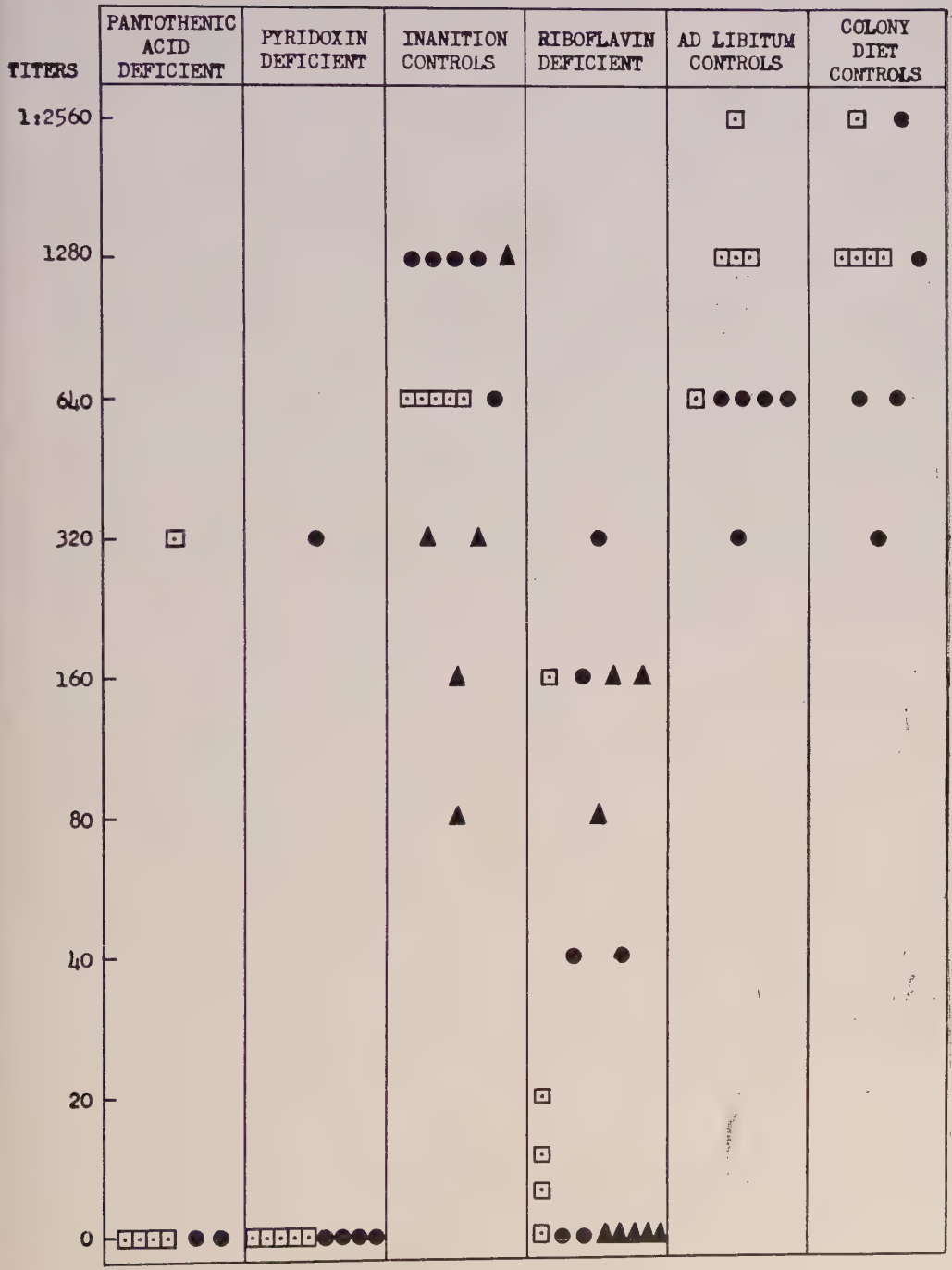
* Group average in grams.
† At the time of bleeding.
‡ Group average in grams.

pill. The basal diet was fed *ad libitum* to all groups except the inanition controls, in which case the daily food intake of each rat was restricted to that consumed during the previous day by its litter mate in the pyridoxin-deficient group. In Series II, the food consumption of the remaining animals was also determined as shown in Table I.

After 7 weeks on experiment, the animals in the 3 vitamin-deficient groups had plateaued in weight and immunization of all animals was instituted. A 10% suspension of washed Group O, Rh positive human erythrocytes in normal saline was given intraperitoneally as antigen. In Series I an initial dosage of 0.5 ml of the red cell suspension was followed by 1 ml inoculations. In Series II, 0.5 ml was also employed for the primary injection, but the dosage for the subsequent injections was 1 ml of red blood cell suspension per 100 g of body weight except that the maximum dosage employed was 2.0 ml. Inoculations were made on alternate days until a total of 6 injections had been given. Five days after the final injection the rats were bled under ether anesthesia and the serum collected. The serums were tested for agglutinin titer within 24 hours after collection. Serial, 2-fold dilutions, beginning with a 1:5 dilution, were made with normal saline. A 0.2 ml sample of each dilution was incubated in 10 x 75 mm tubes at room temperature for one hour with 0.2 ml of a 2% saline suspension of Group O, Rh positive cells of the same origin as those used for the inoculations. At the end of the hour, the tubes were centrifuged lightly and read using a three-plus titer for the endpoint.

Results. The individual hemagglutinin titers are recorded in the scatter chart. It is evident that there was a marked diminution in the content of circulating antibodies in the pantothenic acid and pyridoxin-deficient groups. Thus, only one animal in each of these 2 groups possessed a measurable agglutinin titer. The fact that the agglutinin titers of the rats in the inanition control groups were equal to those of both the control animals which were fed *ad libitum* and the colony diet controls indicates that the lowered content of circulating antibodies in the pantothenic acid and pyridoxin-deficient groups cannot be ascribed to inanition *per se*. This is also borne out by the observation that, despite their poor nutritional state, the riboflavin-deficient rats possessed a higher content of circulating antibodies than either the pantothenic acid or pyridoxin-deficient rats. The content of circulating antibodies in the riboflavin-deficient rats was found to be intermediate between that of the controls and that of the pantothenic acid and pyridoxin-deficient rats. That the increased titer in the riboflavin-deficient group was not due to the

INDIVIDUAL HEMAGGLUTININ TITERS



□—Series I animals.
●—Series II animals.
▲—Comparable animals in which only 3 injections (0.5, 1.0, and 1.0 ml) of cells were used as antigenic stimulus. Inanition controls were pair fed with the riboflavin-deficient group.

higher dosage of antigen per unit of body weight is evident from the results of Series II where the dosage per 100 g of body weight was the same for all three groups. The reproducibility of these observations is demonstrated by the close agreement between the results of Series I and Series II.

Controlled hemolysin tests, using guinea pig complement, were run on serums from Series I. Hemolysin production was consistently low but followed the same pattern as the agglutinin production.

Discussion. A decrease in the content of circulating antibodies in pyridoxin deficiency was reported by Stoerk and Eisen.[2] This work is confirmed in our experiments. Our results, however, differ from those of Stoerk, Eisen, and John[3] with regard to the effects of pantothenic acid and riboflavin deficiencies. While these workers observed a normal antibody titer, we have noted a decreased titer, particularly in the pantothenic acid-deficient rats. The explanation for this difference may lie in the fact that Stoerk, Eisen, and John utilized sheep erythrocytes, whereas we employed human red cells as the antigen. The immunization procedures also differed. Since the agglutinin titers of our normal controls were considerably higher than those of Stoerk and co-workers, it is evident that our immunization procedure with human erythrocytes furnished a far stronger antigenic stimulus for agglutinin production. Sheep erythrocytes, in our hands also, have proved to be a poor stimulus for hemagglutinin production in the rat although they are effective antigens for hemolysin production. It is possible, therefore, that pantothenic acid and riboflavin do not become limiting factors for hemagglutinin production when the antigenic stimulus is of a low order of magnitude. It is also evident from our data that riboflavin is not as critical as pantothenic acid in the production of circulating antibodies.

There is considerable basis for linking pyridoxin and riboflavin to the processes of amino acid metabolism and the role of these vitamins in antibody production may be related to these functions. To our knowledge, a similar role of pantothenic acid in protein metabolism has not been suggested. The present demonstration of the decreased antibody concentration in pantothenic acid deficiency furnishes evidence for the participation of this vitamin in protein metabolism.

As emphasized in the paper of Stoerk, Eisen, and John,[3] the decreased antibody titer in serum is not unequivocal proof for an impairment of antibody synthesis in the tissues. The possible effect of the vitamin deficiency upon the processes of antibody distribution and destruction must also be taken into account before the relationship between vitamins and antibody production can be established.

Summary. 1. Hemagglutinin production in response to inoculation with human erythrocytes has been investigated in pyridoxin, pantothenic acid, and riboflavin-deficient rats. 2. Severe impairment of antibody response was observed in the pantothenic acid and pyridoxin-deficient rats. Variable but low titers were observed in the riboflavin-deficient groups. Consistently high titers were noted in both the inanition controls and the *ad libitum*-fed animals.

We are indebted to Merck and Company for the B vitamins and to the Lederle Laboratories for the synthetic folic acid employed in this study.

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Action of Acridines on Agents of the Psittacosis-Lymphogranuloma Group.*

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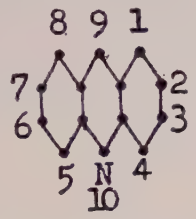
Inhibition of psittacosis infections in mice by trypaflavine (3, 6-Diamino-10-methyl acridinium chloride)† when both drug and



virus were given by the intraperitoneal route has been reported by Mauer.[1] Even when treatment was delayed for 7 days after inoculation of the virus, he observed some therapeutic effect. But in his experiments trypaflavine failed to inhibit cerebral or respiratory infections with psittacosis virus,[1] and Andrewes, King, and van den Ende[2] found that several of the amino acridines and atabrine were without effect on lymphogranuloma venereum virus inoculated into mice by the intracerebral or the respiratory route. Acridines with a nitro substituent at the 3-position inhibit yolk-sac infections with several species of rickettsiae,[3,4] and acriflavine is reported to be rickettsiastatic in chick embryos when the dose is near the toxic level.[3,4] Atabrine, acriflavine, and some other aminoacridines were inactive in mice against respiratory infections with rickettsiae of murine typhus[5] and at concentrations of 1:2,000 were not lethal to this rickettsia *in vitro*. Nitroakridin 3582, or 3 - nitro - 6, 7 - dimethoxy - 9 - (2-hydroxy-3-diethylaminopropylamino) acridine, apparently has an *in vitro* virucidal action or chemoprophylactic effect on influenza virus of type B inoculated into the allantoic sac of chick embryos,[6] but other acridines were ineffective against influenza A in mice.[2] Several aminoacridines and atabrine are reported to inhibit the development of bacteriophage.[7] This paper will describe the action of some of the acridines tried by other investigators, and of a few new derivatives, on the viruses of mouse pneumonitis, cat pneumonitis, meningopneumonitis, and lymphogranuloma venereum.

Materials and Methods. The agent of mouse pneumonitis[8] and the JH strain of the virus of lymphogranuloma venereum[9] were obtained from Dr. Clara Nigg. The cat pneumonitis[10] virus was kindly sent to us by Dr. James A. Baker, and the meningopneumonitis virus,[11] of the strain Cal 10, was originally obtained from Dr. Thomas B. Turner.

Experiments in chick embryos were per-

* These studies were conducted with the support of the International Health Division of The Rockefeller Foundation in cooperation with the California State Department of Public Health.

† In designating the position of substituents in the acridine nucleus, we have used the following system of numbering throughout:

1 Mauer, G., *Zentralblatt Bakt. Abt. Orig.*, 1938, **142**, 279.

2 Andrewes, C. H., King, H., and van den Ende, M., *J. Path. and Bact.*, 1943, **55**, 173.

3 Smadel, J. E., Snyder, J. C., Hamilton, H. L., Fox, J. P., and Jackson, E. P., *Fed. Proc.*, 1946, II, **5**, 254.

4 Smadel, J. E., Snyder, J. C., Jackson, E. B., Fox, J. P., and Hamilton, H. L., *J. Immunol.*, 1947, **57**, 155.

5 Andrewes, C. H., King, H., and Walker, J., *Brit. J. Pharm. and Chemotherap.*, 1946, **1**, 15.

6 Green, R. H., Rasmussen, H. F., Jr., and Smadel, J. E., *Pub. Health Rep.*, 1946, **61**, 1401.

7 Fitzgerald, R. J., and Babbitt, D., *J. Immunol.*, 1946, **52**, 121.

8 Nigg, C., and Eaton, M. D., *J. Exp. Med.*, 1944, **79**, 497.

9 Rake, G., and Jones, H. P., *J. Exp. Med.*, 1942, **75**, 497.

10 Baker, J. A., *J. Exp. Med.*, 1944, **79**, 159.

11 Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1938, **68**, 147.

formed by inoculating dilutions containing between 3 and 10 LD_{50} of egg-adapted virus, as determined by previous titration, into the yolk sacs of 6-day-old embryos and giving the drug by the same route 2 hours later. In certain experiments the injections of drug were repeated 48 and 96 hours after inoculation of the virus. Control eggs received an equivalent amount of physiological saline solution. The specificity of the deaths and the degree of viral multiplication in the yolk sacs of surviving embryos sacrificed on the 8th day were determined by subinoculation of mice by the intranasal route with yolk sac suspension from individual treated and control eggs. When the yolk sac contained little residual virus, the mice developed pulmonary lesions involving one-third or less of the lung tissue. Heavily infected yolk sacs killed part or all of the mice, and in the survivors sacrificed on the 12th day they were found to have produced a 3-plus or 4-plus pulmonary consolidation.

In experiments in the treatment of respiratory infections, mice were inoculated intranasally with dilutions of 10^{-3} to 10^{-4} of the respective mouse-adapted viruses in lung suspension, the infecting dose being adjusted by previous titration so that animals killed on the 4th day had pulmonary consolidation represented by a lesion score‡ of 20 to 35%, while those killed on the 6th day had lesion scores of 50 to 60%. Drugs were given once daily by the intraperitoneal route beginning 2 hours after the inoculation of the virus. Control mice received virus intranasally and saline intraperitoneally concurrently with the treated animals.

The chemical formula, source, and toxic dose of the 8 acridine compounds used in these studies is presented in Table I. For treatment of chick embryos, compounds W243, W138, and A1150 were ground with a small amount of starch to make suspensions

‡ The method of recording pulmonary lesion scores was originally used by Horsfall.[12] A lesion score of 100% represents death with complete pulmonary consolidation. In surviving mice, 80% represents, on the average, 4+ consolidation; 60%, 3+; 40%, 2+; and 20%, 1+.

TABLE I.
Chemical Name and Toxicity of the Acridine Derivatives.

No. or name	Chemical name	Lethal dose mice‖		Lethal dose¶ chick embryos, mg
		acute, mg	delayed, mg	
Acriflavine*	3,6-Diamino-10-methyl acridinium chloride plus 3,6-Diamino acridine HCl	>1.0	4 x 0.5	1.0
Proflavine*	3,6-Diamino acridinium hydrogen sulfate	>1.0	4 x 0.5	1.0
W243†	3-Nitro-6,7-dimethoxy 9-(2-phenyl-4-diethylaminobutylamino) acridine 2 HCl	2.0	4 x 1.0	2 x 0.5
W138†	3-Chloro-7-methoxy 9-(2-phenyl-4-diethylaminobutylamino) acridine 2 HCl	—	4 x 1.0	2 x 1.0
W1889† §	3-Nitro-6,7-dimethoxy 9-(2-hydroxy-3-diethylaminopropylamino) acridine 2 HCl $2H_2O$	5.0	4 x 1.0	2 x 0.5
W10†	3-Chloro-7-methoxy 9-(2-hydroxy-3-diethylaminopropylamino) acridine 2 HCl	5.0	4 x 1.0	2 x 1.0
Atabrine	3-Chloro-7-methoxy 9-(1-methyl-4-diethylaminobutylamino) acridine 2 HCl	2.5	4 x 1.5	2 x 1.0
A1150‡	3-Nitro-9-aminoacridine HCl	1.0	4 x 0.5	2 x 0.5

* Purchased from National Aniline Division, Allied Chemical and Dye Co., Inc.
† Given by Dr. M. L. Tainter, Sterling-Winthrop Research Institute.
‡ Given by Dr. D. L. Tabern, Abbott Laboratories.
§ Same formula as Nitroakridin 3582. See references 3 and 6.
‖ Weight 15-18 g. Delayed lethal dose represents amount of drug given on 4 successive days.
¶ Two doses given 48 hours apart.

TABLE II.
Effect of Acridines on Yolk Sac Infections with the Cat Pneumonitis Virus in Chick Embryos.

Drug	Dosage mg/egg	Dosage No.	Mortality ratios and degree of infection on 8th day* Treated egg	Control eggs	% reduction of Mortality	% reduction of Heavy infection
Acriflavine	.3	2	0/11 (1)†	15/16 (1)†	—*100*	—*91*
	.3	1	0/9 (2)		—*100*	—*78*
	.2	1	0/6 (4)	8/8	—*100*	—33
	.1	1	8/9 (1)		— 11	0
Proflavine	.3	2	4/5 (1)	6/7 (1)	— 7	0
W243	.3	3	1/14 (2)	15/18	— *91*	—*74*
	.3	1	0/8	12/12	—*100*	—*100*
	.1	1	3/4 (1)		— 25	0
W138	.3	2	7/7	3/4	0	0
W1889	.3	1	0/8		—*100*	—*100*
	.2	1	0/9 (2)	8/8	—*100*	— *78*
	.1	1	4/8 (1)		— *50*	— 38
Atabrine	2.0	1	8/8	8/8	0	0
	.5	3	6/9 (2)	6/7	— 22	0
A1150	.2	2	8/20 (10)	17/18	— *58*	— 4

* 3 to 10 LD_{50} of virus in each experiment.
†Figures in parentheses represent number of surviving eggs which were found to be heavily infected by subinoculation to mice and production of pulmonary lesions involving, on the average, over half of the lungs. Lesion scores in mice inoculated from remainder of living eggs were less than 30%.

in physiological saline which were then autoclaved at 10 lb pressure for 10 minutes. Solutions of the remaining drugs were sterilized by filtration through bacteria-retaining fritted glass discs. When used in mice the solutions or suspensions were not sterilized. In the table the lethal dose is given as the least amount which killed more than half of the mice or chick embryos. The delayed lethal dose for mice is given as the daily dose in milligrams injected intraperitoneally on 4 successive days. In chick embryos, 2 doses separated by an interval of 48 hours were inoculated into the yolk sac.

Results in chick embryos infected with the virus of cat pneumonitis. Seven acridine derivatives were tested in chick embryos against the virus of cat pneumonitis as shown in Table II. The results are evaluated in terms of mortality of the embryos by the 8th day after inoculation, at which time most of the controls were dead. In the 4th and 5th columns the figures in parentheses represent the number of additional eggs in each group which survived with heavy infection, as determined by the mouse test. These probably would have died before the time of hatching. The remainder of the surviving chick embryos had little residual virus at 8 days or none detectable by subinoculation of mice.

The last 2 columns of the table show the percentage reduction of mortality in the treated eggs as compared with the controls, and the percentage reduction in the incidence of heavy infection. The formula 100 (-1+T/C) is used; T represents the percent mortality in the treated eggs, and C the percent mortality in the controls. For estimating the percentage reduction in heavy infections, the figures in parentheses (columns 4 and 5) were added to the numerator of each fraction and the calculations were again carried out with the formula given above. Reductions of 40% or over are italicized, and are considered to be significant.

Acriflavine and the 2 nitroacridines, W243 and W1889, were effective in single doses of 0.2 to 0.3 mg, or 1/3 to 1/5 of the lethal dose for chick embryos. With the possible exception of W1889, no appreciable effect was

TABLE III.
Effect of Acridines on Respiratory Infection with Cat Pneumonitis Virus in Mice.

Drug	Daily drug dosage mg/mouse	Daily drug dosage No.	Mice No.	Mice Day killed	Pulmonary lesion scores in % Treated mice*	Pulmonary lesion scores in % Controls	%† reduction in pulmonary consolidation
Acriflavine	0.2	4	19	4	21	37	*—43*
		5	19	6	52	57	— 9
Proflavine	,,	4	18	4	34	37	— 8
		5	19	6	57	57	0
W243	0.4	4	39	4	9.5	32	*—70*
		5	23	6	27	56	*—52*
W138	0.5	4	16	4	36	42	—14
		5	8	6	60	67	—10
W1889	0.4	4	12	4	2.5	24	*—90*
		5	30	6	5.0	56	*—91*
W10	0.5	5	22	6	44	48	— 8
A1150	0.2	4	26	4	21	23	— 9
		5	12	6	47	50	— 6
Penicillin G	7.9‡	4	18	4	8.9	32	*—72*

* Dilutions of virus 2×10^{-3} to 5×10^{-4} inoculated intranasally.
† 100(—1 + T/C).
‡ 1520 units of penicillin G per mg or 12,000 units per day given in 3 doses daily.

found with doses of 0.1 mg of these substances. In other preliminary experiments, some inhibitory effects were observed with the 3 drugs even when treatment was delayed for 24 or 48 hours after inoculation of the virus. The 3-nitro-9-aminoacridine, A1150, which differs from the others in having no side chain and no methoxy groups (see Table I) caused some reduction in the mortality, but was much less inhibitory than the other 3 drugs when given in equivalent dosage. Proflavine, in contrast to acriflavine, failed to influence the mortality of embryos infected with the cat pneumonitis virus under the conditions of these experiments. A slight inhibitory effect of proflavine on virus growth was demonstrable when the chick embryos were sacrificed 5 days after inoculation and the yolk sacs tested in mice. (The results of this test are not included in Table II.) The two acridines, W138 and atabrine, differ from W243 and W1889, respectively, by the presence of a single methoxy group and the substitution of a chlorine atom for the 3-nitro group. As may be seen in Table II, these 2 chloroacridines had no effect on the virus of cat pneumonitis in chick embryos when given in amounts considerably larger than the therapeutic dose of the corresponding nitroacridines.

Results in mice with cat pneumonitis virus. Mice inoculated by the intranasal route with the virus of feline pneumonitis were given daily doses of the acridine compounds in amounts representing ½ to ⅖ of the toxic dose. The results are presented in Table III. The 3-nitroacridines (W243 and W1889) which were most effective in chick embryos also caused a significant reduction in the pulmonary consolidation of the mice, while the closely related 3-chloroacridines (W138 and W10) were inactive. Acriflavine, proflavine, and 3-nitro-9-aminoacridine (A1150) were about twice as toxic for mice as the other drugs and were, therefore, used in half the amount. The result with acriflavine in mice sacrificed 4 days after inoculation was of borderline significance, and the other compounds had no effect at the maximum tolerated dosage.

For comparison, a group of mice infected with the same virus received 12,000 units of penicillin per day divided into 3 doses of 4,000 units each and given at intervals of 7, 7, and 10 hours. The resulting inhibition of pulmonary consolidation in the mice was the same or slightly less than that caused by the nitroacridines. It should be noted that the acute lethal dose of the crystalline penicillin G used in these experiments was greater than 50,000 units.

Mouse pneumonitis, lymphogranuloma ven-

TABLE IV.
Action of Acridines on the Viruses of Mouse Pneumonitis, Lymphogranuloma Venereum, and Meningopneumonitis in Mice and Chick Embryos.

Drug	Virus (10 LD_{50})	Results in chick embryos*			Results in mice†		
		Dose,* mg	No. tested	% change	Dose, mg	No. tested	% change
Acriflavine	Mouse Pn.	0.3	16	— 35	0.2	40	+10
	LGV	"	6	*—100*	"	24	*—40*
	Meningopn.	"	19	*— 86*	"	24	—14
W243	Mouse Pn.	"	20	— 16	0.4	31	+19
	LGV	0.2	13	*— 54*	"	28	+ 6
	Meningopn.	"	16	*— 67*	"	27	— 6
W1889	Mouse Pn.	"	10	*— 70*	0.3	24	—18
	LGV	"	9	*— 87*	"	24	*—46*
	Meningopn.	"	9	*— 89*	"	24	*—40*

* Drug given to chick embryos 2 and 48 hours after virus. Results expressed as reduction of incidence of heavy yolk sac infection in embryos harvested at 8 days when 75 to 100% of control embryos were heavily infected (see Table II).

† Mice received drug daily as in Table III. Results averaged for groups killed at 4 and 6 days, expressed as percentage reduction or increase in lesion scores of treated mice as compared with controls.

ereum, and meningopneumonitis in chick embryos and mice. A summary of the results of preliminary experiments with 3 other viruses of the group is presented in Table IV. The drugs most effective against the cat pneumonitis virus, acriflavine and the nitroacridines W243 and W1889, were used in amounts and dosage schedules comparable to those listed in Tables II and III. In chick embryos, all 3 drugs definitely inhibited the growth of the viruses of lymphogranuloma venereum and meningopneumonitis, the effects being similar to those obtained with the agent of feline pneumonitis when expressed as reduction in incidence of heavy infection (see last column of Table II). Against the virus of mouse pneumonitis acriflavine gave results which were just below the level of significance and W243 showed no therapeutic activity. In other experiments not included in Table IV, retarded growth of this virus in embryos treated with acriflavine or W243 was observed when the yolk sacs were harvested at 5 days. The other nitroacridine (W1889) caused more definite inhibition of the growth of the mouse pneumonitis virus in chick embryos.

In mice the inhibition of respiratory infections with the 3 viruses mentioned above was less striking than that occurring in infections with the cat pneumonitis virus. None of the drugs produced appreciable reduction in the pulmonary consolidation resulting from the mouse pneumonitis virus. With one nitroacridine, W1889, results of borderline significance were obtained in tests against the viruses of lymphogranuloma venereum and meningopneumonitis, but the other nitroacridine, W243, had no effect on these 2 viruses in the lungs of mice. Acriflavine showed questionable activity against pulmonary infections with the virus of lymphogranuloma venereum and none against the agent of meningopneumonitis.

Discussion. The results of these experiments suggest that replacement of the NO_2 group at the 3-position on the acridine nucleus by Cl results in loss, or marked diminution, of the inhibitory action against the virus of feline pneumonitis. Since the relatively simple compound 3-nitro-9-aminoacridine had some inhibitory action in chick embryos, it seems that the other substituents such as the 6- or 7- methoxy groups and the 9-dialkylaminoalkylamino side chain are not absolutely essential to the antiviral activity. The possible role of the side chain at the 9- position must be investigated further, because the compounds with this substituent seemed to be somewhat more active on weight basis than the simple 3-nitro-9-aminoacridine. Also, the preliminary results in Table IV suggest a slightly wider range of activity for the compound with the 9-(2-hydroxy-3-diethylaminopropylamino) substituent (W1889) as compared with the 9-(2-phenyl-4-diethylamino-

butylamino) group (W243).

It remains to be seen whether acriflavine acts in the same manner as the nitroacridines. The active constituent of acriflavine seems to be the 10-methyl acridinium chloride (see Mauer[1]). Proflavine, which is the sulphuric acid salt without the 10-methyl group, was much less inhibitory for the feline pneumonitis agent in chick embryos. Its activity against other viruses of the group is under investigation.

The relative resistance of the mouse pneumonitis virus to acriflavine and to one of the nitroacridines is surprising in view of the high sensitivity of this agent to penicillin[13,14] and sulfonamides.[15] The feline pneumonitis and meningopneumonitis viruses which are, on the other hand, relatively resistant to penicillin and are not affected in mice or chick embryos by sulfonamides are quite readily inhibited in chick embryos by acriflavine and the nitroacridines. The observed differences in the chemotherapeutic spectrum for penicillin, sulfonamides, and acridines suggest interesting variations in the enzymatic or metabolic constitution of the agents of the psittacosis-lymphogranuloma group. The 3 classes of substances probably act on 3 different constituents of the viruses under consideration.

The results in mice tended to parallel those in chick embryos, but on a lower scale of activity so that less conclusive results were obtained. Effective inhibition of respiratory infections was observed only with the 2 nitroacridines against the cat pneumonitis agent, but these 2 substances were, on a weight basis, more effective than penicillin against this virus. Because of the high toxicity of acridines and their failure to show general activity against the psittacosis-lymphogranuloma group in mice, no claim for their therapeutic usefulness can be made at the present time.

Summary. Acriflavine; 3-nitro-6, 7-dimethoxy 9 - (2-phenyl-4-diethylaminobutylamino) acridine; and 3-nitro-6, 7-dimethoxy 9 - (2-hydroxy - 3-diethylaminopropylamino) acridine inhibited yolk sac infections of chick embryos with the agents of feline pneumonitis, lymphogranuloma venereum, and meningopneumonitis. The first two compounds were less active against the virus of mouse pneumonitis, but the last-named inhibited this agent in chick embryos.

Proflavine, atabrine, and drugs closely related to the above-mentioned nitroacridines, except for substitution of Cl for NO_2, had no significant inhibitory action. 3-nitro-9-aminoacridine was intermediate in its effect.

Respiratory infections in mice caused by the agent of feline pneumonitis were retarded by the two nitroacridines, but these drugs showed slight or no effect in mice against intranasal infection with the agents of mouse pneumonitis, lymphogranuloma venereum, and meningopneumonitis.

12 Horsfall, F. L., Jr., *J. Exp. Med.*, 1939, **70**, 209.

13 Meiklejohn, G., Wagner, J. C., and Beveridge, G. W., *J. Immunol.*, 1946, **54**, 1.

14 Eaton, M. D., Dozois, T. F., van Allen, A., Parish, V. L., and Schwalm, S., *J. Immunol.*, in press.

15 Eaton, M. D., and Hanford, V. L., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 63.

16012

Effect of Egg Yolk on Release of Antigen from Rickettsiae.

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The existence of a soluble antigen which is released on treatment of certain rickettsiae with ether has been well established with respect to *Rickettsia prowazeki*,[1] *R. rickettsii*,[2] and *R. akari*.[3,4] That this antigen, in the case

1 Topping, N. H., and Shear, M. J., *Pub. Health Rep.*, 1944, **59**, 1671.

of typhus fever rickettsiae at least, is complete is shown by its activity both *in vitro* and *in vivo*. *In vitro* it is active as a complement fixation test antigen[1] and a precipitin test antigen,[5] and *in vivo* it is able to immunize guinea pigs against the disease[1] with the formation of antibodies demonstrable in the complement fixation test,[1] neutralization and precipitin tests.[6] By the use of the electron microscope, it has been demonstrated that the antigen probably arises from a capsule-like structure on the surface of the organism, and that it exists as a suspension of submicroscopic particles of the capsular substance.[7]

When purified suspensions of rickettsiae were prepared without the use of ether and subsequently shaken with ether[10] little soluble antigen could be demonstrated. When a normal yolk sac suspension was added to the purified rickettsiae, soluble antigen was released with ether. Further experiments are presented here to show that some component of egg yolk plays a part in facilitating the release of the soluble antigen from rickettsiae by ether treatment at room temperature.

The rickettsial suspensions used in these experiments were prepared from infected yolk sacs of chicken embryos. By differential centrifugation efforts were made to remove some of the yolk sac tissues and soluble proteins. The rickettsiae were finally suspended in a limited quantity of saline or distilled water containing 0.1% formalin. In the antigen release experiments generally 0.2 ml of the rickettsial suspension was mixed with 0.6 ml of diluent, either the universal buffer of

TABLE I.

Effect of Normal Egg Yolk on Release of Antigen from Rickettsiae. Homologous convalescent guinea pig serum was used in each instance.

Soluble antigen from	Conc. of soluble antigen (as original yolk sac) %	pH	Diluent	Complement fixation results Dilutions of soluble antigen 1:4	1:8	1:16	1:32	1:64	1:128	Antigen control 1:4
*R. prowazeki** (Breinl strain)	25	6.14	2.5% yolk	++++	++++	++++	++	—	—	—
		6.10	Buffer only	+++	—	—	—	—	—	—
*R. mooseri** (Wilmington strain)	15	6.1	2.5% yolk	++++	++++	++++	++++	++	—	—
		6.0	Buffer only	+	—	—	—	—	—	—
R. rickettsii† (Bitter Root strain)	50	6.2	2.5% yolk	++++	+++	+	—	—	—	—
		5.6	Buffer only	+++	trace	—	—	—	—	—
R. akari‡ (Wild mouse strain)	25	6.0	2.5% yolk	++++	++++	++++	trace	—	—	—
		5.95	Buffer only	+++	++++	trace	—	—	—	—
R. burneti† (Italian strain)	50	6.14	2.5% yolk	—	—	—	—	—	—	—
		6.10	Buffer only	—	—	—	—	—	—	—

* Rickettsiae were washed with salt solution pH 7.6 and distilled water according to the method of Shepard and Topping.[10]
† Rickettsiae were sedimented once, then resuspended in saline.
‡ Rickettsiae were sedimented two times and resuspended in distilled water.

2 Plotz, H., Reagan, R. L., and Wertman, K., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 173.

3 Huebner, R. J., Stamps, P., and Armstrong, C., *Pub. Health Rep.*, 1946, **61**, 1605.

4 Huebner, R. J., Jellison, W. L., and Pomerantz, C., *Pub. Health Rep.*, 1946, **61**, 1677.

5 Shepard, C. C., and Topping, N. H., *Nat. Inst. Health Bull., No. 183*, Government Printing Office, Washington, 1945, p. 87.

6 Shepard, C. C., *Nat. Inst. Health Bull. No. 183*, Government Printing Office, Washington, 1945, p. 93.

7 Shepard, C. C., and Wyckoff, R. W. G., *Pub. Health Rep.*, 1946, **61**, 761.

Michaelis[8] or the same buffer containing 2.5% of normal yolk from fresh chicken eggs. To each mixture were added 1.5 ml of anaesthetic ether. The two phases were then shaken together vigorously 30 times. The aqueous layer was drawn off, freed of ether with vacuum and centrifuged at 4000 r.p.m. for one hour to sediment the rickettsiae. The supernatants were tested for antigen content by the complement fixation test[9] using 4 units of homologous convalescent guinea pig serum. The results of the tests with five species of rickettsiae are shown in the table.

The readings in the table show that in the presence of 2.5% yolk higher titers were obtained with *R. prowazeki, R. mooseri, R. akari,* and *R. rickettsii.* With *R. burneti* no soluble antigen could be detected.

When normal yolk was diluted 10 times with 0.85% sodium chloride solution and extracted with 2 volumes of ether, it was found that the water soluble constituents in the aqueous layer had the same degree of activity in releasing soluble antigen from rickettsiae as the original yolk.

Although it will be noted that some antigen was released without the addition of yolk, such release may have been due to the amount of yolk remaining with the preparation of rickettsiae, since the more carefully purified Breinl and Wilmington suspensions showed little release except when yolk was added. It has been noted that the increase in titer obtained by adding yolk is variable unless the rickettsiae are carefully washed. Yolk sacs are known to carry more or less yolk after harvest and it is evident that the amount of adherent yolk can effect the complement fixation titer of an ether extracted antigen. For consistent results it has been found advantageous to add 5 or 10% normal egg yolk to all method I and method II antigens[11] for use in complement fixation tests.

In the preparation of Rocky Mountain spotted fever antigens by method I or II,[11] the addition of yolk is especially valuable[12] since the growth of *R. rickettsii* in yolk sacs is poor, and antigen release is frequently not marked.

Summary. The addition of as little as 2.5% egg yolk to suspensions of yolk sacs infected with the rickettsiae of epidemic and endemic typhus fever, Rocky Mountain spotted fever, and rickettsialpox (*R. prowazeki, R. mooseri, R. rickettsii,* and *R. akari*) prior to treatment with ether has resulted in antigens with enhanced titers as measured by the complement fixation test.

8 Michaelis, L., *Biochem. Z.*, 1931, **234**, 139.

9 Bengtson, I. A., *Pub. Health Rep.*, 1944, **59**, 402.

10 Shepard, C. C., and Topping, N. H., *J. Immunol.*, 1947, **55**, 97.

11 Topping, N. H., and Shepard, C. C., *Pub. Health Rep.*, 1946, **61**, 701.

12 Unpublished experiments.

16013

Renal Hemodynamic Effects of Adrenaline and "Isuprel": Potentiation of Effects of Both Drugs by Tetraethylammonium.

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Vasodepressor drugs have been prepared which are built on the chemical nucleus of sympatheticomimetic compounds. The pharmacological properties of one of these, the N-isopropyl homologue of adrenaline, called "Isuprel" (1-(3[1],4[1]-dihydroxyphenyl)-2-isopropylaminoethanol hydrochloride) have been described by Lands and his colleagues.[1] The suggestion has been made that the activity of

1 Lands, A. M., Nash, V. L., McCarthy, H. M., Grainger, H. R., and Dertinger, B. L., *J. Pharm. Exp. Therap.*, 1947, **89**, 110.

TABLE I.
Effects on Renal Function of Adrenaline, Tetraethylammonium, and "Isuprel."

Dog	Per.	C_{PAH} cc per min	C_{CR} cc per min	FF	R	B P mm Hg	Pulse	Duration min
4-9 14 kg	1-2	208	62.6	0.30	0.27	149	110	20
			Adrenaline 0.1 cc 1/1000 i.v.					
	3-4	198	63.7	0.32	0.29	152	130	20
			Et_4N 2 cc 10% i.v.					
	5-6	254	69	0.27	0.18	129	135	20
			Adrenaline 0.1 cc 1/1000 i.v.					
	7	163	51	0.31	0.39	161	100	10
	8	197	60	0.30	0.22	128	112	10
6-3 18 kg	1-2	178	46	0.26	0.24	141	98	20
			Adrenaline 0.1 cc 1/1000 i.v.					
	3-4	130	47.4	0.36	0.31	140	100	20
			Et_4N 2 cc 10% i.v.					
	5-6	145	40.4	0.28	0.27	134	130	20
			Adrenaline 0.1 cc 1/1000 i.v.					
	7-8	111	32.5	0.29	0.46	158	84	18
4-9 14 kg	1-2	149	56	0.37	0.32	149	110	20
			"Isuprel" 0.2 cc 1/1000 i.v.					
	3	144	58	0.40	0.29	150	145	10
	4	175	58	0.33	0.20	123	150	31
	5	169	40	0.24	0.29	147	120	20
			Et_4N 2 cc 10% i.v.					
	6	170	53	0.31	0.28	147	140	30
	7	129	49	0.38	0.33	140	130	20
			"Isuprel" 0.2 cc 1/1000 i.v.					
	8	68	24	0.35	0.17	83	148	15
	9	278	46	0.31	0.10	86	176	20
	10	251	46	0.34	0.19	107	172	12
3-7 12 kg	1-3	218	74	0.34	0.21	135	55	30
			"Isuprel" 0.1 cc 1/1000					
	4-5	202	61	0.30	0.19	122	80	20
			Et_4N 2 cc 10% i.v.					
	6-7	236	58	0.25	0.14	114	128	30
			"Isuprel" 0.1 cc 1/1000 i.v.					
	8	223	63	0.28	0.10	93	140	20
	9	231	68	0.29	0.20	136	112	20

Dog number and body weight are indicated in the left hand column. Per. = intervals of urine collection. C_{PAH} = plasma clearance of *p*-aminohippurate. C_{CR} = plasma clearance of creatinine. FF = filtration fraction (C_{CR}/C_{PAH}). R = renal resistance as sum of R_A and R_E calculated by the method of Lamport. B.P. = average of arterial pressure during the time of the observation. Pulse = average of pulse rate during time of observation. Duration of the observation is indicated in minutes.

this and similar compounds resembles that posited for sympathin I, since many of its effects contrast with those of adrenaline and with those credited to sympathin E. Since adrenaline is a renal vasoconstrictor, it seemed likely that 'Isuprel' might be a renal vasodilator. One of the purposes of this report is to describe the effect of injection of "Isuprel" on the renal circulation.

Another aspect rests on the observation of Page and Taylor[2] that the effect on arterial pressure of adrenaline and other pressor drugs is increased by pre-treatment with tetraethylammonium (Et_4N). We demonstrate below that potentiation of the action of adrenaline by pre-treatment with Et_4N is reflected in renal circulation as well as arterial pressure and (b) that the depressor, cardiac accelerator and renal vasodilator properties of "Isuprel" are similarly increased.

Procedures. Six experiments were done, 3 each with adrenaline and 'Isuprel', on trained conscious dogs. In these renal plasma clearances of p-aminohippurate and creatinine

2 Page, I. H., and Taylor, R. D., *Science*, 1947, **105**, 622.

were determined. The plasma clearance of p-aminohippurate is taken as equivalent to renal plasma flow and the plasma clearance of creatinine as equivalent to the rate of glomerular filtration. Femoral arterial pressure was measured from a mercury manometer and inlying needle. Each experiment consisted of (a) 2 or 3 control periods of clearance measurement; (b) observations after intravenous infusion of small doses of either adrenalin or "Isuprel"; (c) observations after infusion of Et_4N and (d) observations after a second injection of either of the 2 sympatheticomimetics. One experiment was done in a dog anesthetized with pentobarbital. In this only "Isuprel" and Et_4N were injected.

Results. Observations in 2 experiments on conscious dogs in which adrenaline and Et_4N were injected and in 2 in which "Isuprel" and Et_4N were tested are summarized in Table I. The course of one experiment of each type is shown graphically in Fig. 1. Fig. 2 demonstrates the effect on arterial pressure of injections of "Isuprel" Et_4N in the anesthetized dog.

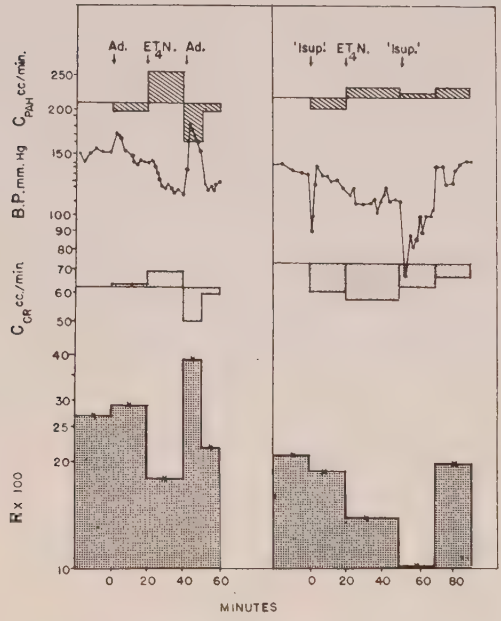

FIG. 1.

Graphic summary of 2 experiments. C_{PAH} = renal plasma clearance of *p*-aminohippurate. C_Cr = plasma or creatinine clearance. B. P. mm Hg = arterial pressure and is shown from repeated observations of femoral arterial pressure. R = renal resistance, and, for convenience, is shown times 100. The experiment on the left was done on dog No. 4-9, body weight 14 kg, and corresponds to the first experiment in Table I. The experiment on the right was done on dog No. 3-7 and corresponds to the last experiment in Table I.

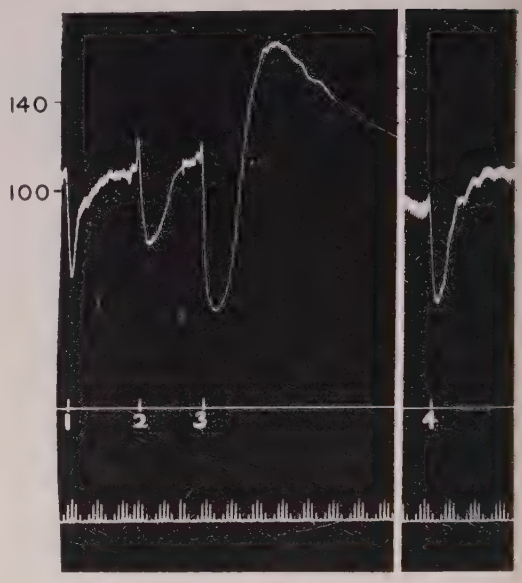

FIG. 2.

Effect on arterial pressure of injection of "Isuprel" and Et_4N in an anesthetized dog, of 10.7 kg body weight. At 1 there was injected 0.2 cc of 1/20,000 "Isuprel." At 2 the animal was given intravenously 10 mg of tetraethylammonium chloride per kg body weight. At 3 the dose of "Isuprel" was repeated. At 4, 30 minutes after injection of tetraethylammonium, the same dose of "Isuprel" was again injected.

Adrenaline. The first injection of adrenaline caused a slight, transient increase of arterial pressure with some increase in renal vascular resistance as calculated by the method of Lamport.[3] Injection of Et_4N slightly decreased renal resistance and arterial pressure in 2 of the 3 experiments. A second injection of adrenaline 20 minutes after injection of Et_4N resulted in increases of arterial pressure and renal vascular resistance which were much greater than the responses to the first injection.

"Isuprel." The first injection caused transient hypotension and tachycardia and a modest decrease in renal resistance. Injection of Et_4N caused again slightly decreased ar-

[3] Lamport, H., *J. Clin. Invest.*, 1943, **22**, 461.

terial pressure and renal resistance in 2 of 3 experiments. A second injection of "Isuprel" resulted in hypotension, tachycardia and decreased renal resistance which were greater in extent and duration than the effects observed before injection of Et_4N. In the anesthetized dog, the vasodepressor effect of "Isuprel" is shown to be increased by injection of Et_4N. The augmentation is demonstrable at 5 but not at 30 minutes after injection of Et_4N. The reactive pressor effect of "Isuprel" after injection of Et_4N was observed in other experiments on anesthetized dogs.

Comment and Summary. The increased pressor responsiveness to adrenaline caused by injection of Et_4N is shown to be associated with an increase in renal vasoconstriction. The depressor response to injection of "Isuprel," the N-isopropyl homologue of adrenaline, is shown to be associated with renal vasodilation. The depressor, cardioaccelerator and renal vasodilator effects of injection of "Isuprel" are augmented by prior injection of tetraethylammonium. On the assumption that the effects of tetraethylammonium are due to blocking of autonomic ganglia,[4] we conclude that inhibition of these ganglia increases vascular responsiveness to typical vasoconstrictor and vasodilator sympatheticomimetics.

We acknowledge the donation of "Isuprel" hydrochloride by Dr. Earl Burbidge, Frederick Stearns Co., Inc.; of tetraethylammonium chloride ("Eatmon") by Dr. E. C. Vonder Heide, Parke, Davis and Co., Inc.; of *p*-aminohippurate by Drs. Karl Beyer and W. Boger, Sharp and Dohme, Inc., and the skillful assistance of William West.

[4] Acheson, G. H., and Moe, G. K., *J. Pharm. Exp. Therap.*, 1945, **84**, 189.

16014 P

Pharmacology of Dibenzyl-β-Chloroethylamine Hydrochloride (Dibenamine).*

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Recently Nickerson, Goodman and Smith,[1,2,3] Raab and Humphreys,[4] Acheson and co-workers,[5] Haimovici[6] reported their experiments with dibenzyl-β-chloroethylamine hydrochloride (dibenamine).

We wished to investigate further the pharmacology of dibenamine and the mechanism of transmission of sympathetic nerve impulses.

Methods. The experiments have been carried out on dogs under chloralosane anesthesia. The blood pressure was registered from a femoral artery. In some experiments the peripheral vasomotor reactions were recorded by means of the 3-manometers-method.[7]

Results. I. *Effects of Dibenamine on blood pressure and heart rate.* Dibenamine, from 1 to 5 mg/kg, does not induce a noticeable change in blood pressure, nor any variations of heart rate. Doses of 10 to 15 mg/kg produce a slight and transient hypotension, but no direct change of heart rate. Subsequent injections of dibenamine do not produce the some hypotension. A primary, slight increase

* This investigation was made with the assistance of grants from the Ella Sachs Plotz Foundation, New York.

[1] Nickerson, M., and Goodman, L. S., *Fed. Proc.*, 1946, **5**, 194.

[2] Nickerson, M., Smith, S. M., and Goodman, L. S., *Fed. Proc.*, 1946, **5**, 195.

[3] Nickerson, M., and Goodman, L. S., *J. Pharm. Exp. Therap.*, 1947, **89**, 167.

[4] Raab, W., and Humphreys, R. J., *J. Pharm. Exp. Therap.*, 1946, **88**, 268.

[5] Acheson, G. H., and co-workers, *Fed. Proc.*, 1947, **6**, 305.

[6] Haimovici, H., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 486.

[7] Nolf, P., *Bull. Acad. Roy. Belg.*, 1902, p. 895.

of blood pressure has been recorded in some experiments.

II. *Localization of the hypotensive action of dibenamine.* No noticeable changes in blood pressure have been observed after injection of dibenamine into the carotid artery (to control a possible central action), while small quantities of dibenamine injected into the peripheral circulation produced an immediate local vasodilatation.

III. *Effects of Dibenamine upon the carotid sinus vasomotor reflexes.* Dibenamine (0.5 to 15 mg/kg) does not produce a marked change of the pressor and depressor carotid sinus reflexes on the arterial blood pressure; same doses, however, reverse the pressor action of epinephrine into a vasodepressor one. Only 60 to 90 minutes after the injection of high doses of dibenamine, a depression, but not a reversal of the carotid sinus vasopressor reflexes could be observed.

IV. *Effects of Dibenamine on hypertensive substances.* Reversal of the vasopressor action of epinephrine appears after administration of at least 10 mg/kg dibenamine. But dibenamine does not suppress or reverse the epinephrine tachycardia. Small quantities of dibenamine, injected into the circulation of the leg, induce a strictly localized reversal of the vasoconstrictor effect of epinephrine.

Ten to 15 mg/kg dibenamine do not reverse the hypertensive action of nicotine in the atropinized dog. Larger quantities decrease slightly, but never reverse, the vasopressor effects of nicotine.

In atropinized dogs, with normal adrenal glands, 10 to 15 mg/kg dibenamine convert the vasopressor action of acetylcholine into a vasodilator response.

The vasoconstrictor effects of ephedrine and pituitrin are scarcely altered by dibenamine.

Ten to 15 mg/kg dibenamine convert the asphyxia hypertension into an hypotension.

V. *Effect of Dibenamine on sympathetic nerve stimulation.* In dogs, with adrenal glands, after injection of 10 to 15 mg/kg dibenamine, the hypertensive response of splanchnic nerve stimulation is converted into a vasodilatation, after an initial rise of the blood pressure.

In dibenamine-treated dogs, after adrenalectomy, the stimulation of the splanchnic nerve produces a monophasic or no rise of the blood pressure; no reversal of this neurovasopressor effect has been observed.

After injection of dibenamine, the general reflex vasoconstrictor response, induced by stimulation of the central end of the vagus, is not converted into a vasodilatation.

Summary. Experiments on dogs showed that dibenamine:

1. Induces a slight fall of arterial pressure; this hypotension depends mainly on a peripheral vasodilator action.

2. Has no direct effect upon the heart rate.

3. Produces no lasting decrease of the carotid sinus vasopressor reflexes; it never induces a reversal of these reflexes, while the same dose reverses completely the action of epinephrine on blood pressure and blood vessels.

4. Does not affect the tachycardic action of epinephrine.

5. Has no marked effect upon the hypertensive properties of nicotine, ephedrine and pituitrin.

6. Produces a reversal of the asphyxia hypertension.

7. Induces, in atropinized dogs, a reversal of the vasopressor action of acetylcholine.

8. May decrease, but does not produce a reversal of the vasomotor responses of sympathetic origin.

9. Is primarily a powerful adrenolytic, but a weak sympathicolytic agent.

16015 P

Mitoses in the Livers of Rats Treated with Thiourea.

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The purpose of the present communication is to report the remarkable effect of large doses of thiourea on the liver of rats. This effect is manifested by the appearance of numerous mitoses in the liver cells.

Material and Methods. Thirty-six young male albino rats, weighing between 70 and 100 g were used in these experiments. Four-tenths of a gram of thiourea in a 10% aqueous solution was injected by the intraperitoneal route at daily intervals. Six rats died a few hours after the first injection, 3 succumbed after the second and 8 after the third. Fifteen rats survived 3 injections and were sacrificed 2 to 12 hours after the last injection. Of the remaining 4 rats 3 were sacrificed after 4, and 1 after 7 injections.

The livers were fixed in Carnoy's fluid or in Zenker's solution. Paraffin-celloidin sections 5μ in thickness were stained with hematoxylin-eosin or with Heidenhain's hematoxylin.

Results. In the 23 rats which succumbed or which were sacrificed after 3 daily injections of thiourea, the gross appearance of the livers was normal. Microscopically, there was central congestion of the sinusoids and slight to moderate vacuolization of the liver cells in the central parts of the lobule, as well as slight alterations in the lining of the central vein. The outstanding feature was the presence of all stages of mitotic figures in the liver cells (Fig. 1). Mitoses were found in all animals receiving this treatment, but there were variations in the number of mitoses from rat to rat. In 22% of the treated rats the number of mitoses was extremely high (12 to 18 mitoses in each microscopic field);* in 26% they were numerous (6 to 12 mitoses in each microscopic field); a moderate number of mitoses was present in 35% of the animals (1 to 6 mitoses in each microscopic field) and in 17% of the treated rats the number of mitoses was small (one mitosis being found in 1 to 3 microscopic fields).

The configuration of the mitoses was in most cases completely normal and the different phases of the mitotic processes were encountered in the usual proportions. In a number of livers abnormal mitotic figures were found in moderate or appreciable numbers. The pathological mitoses were characterized by clumping or scattering of the chromosomes. In some experiments mitosis with widely scattered chomosomes was the only type encountered. At the result of chromosomal scattering, liver cells with bizarre nuclear structures appeared at times: some loaded with minute nuclei and others with single or multiple large, lobated nuclei. The multinuclear liver cells were often exceptionally large.

In rats which died or were sacrificed within 24 to 31 hours after the first injection no mitoses were found in the liver cells. After 4 injections, *i.e.*, 72 to 96 hours after the start of the experiment, the number of mitoses was considerably lower than after 3 injections. In one rat examined after 7 injections no mitoses were found. It is thus evident that the mitotic effect is not immediate, but appears only after a certain latent period. The extensive mitotic activity of the liver seems to be limited to a time period of 48 to 72 hours after the first injection.

Comment. The occurrence of mitoses in the liver or in other visceral organs, excepting for the thyroid gland, following treatment with thiourea has not as yet been noted. In the thyroid glands of rats, receiving thiourea,

* The mitotic counts were made in microscopic fields of 0.4 mm diameter.

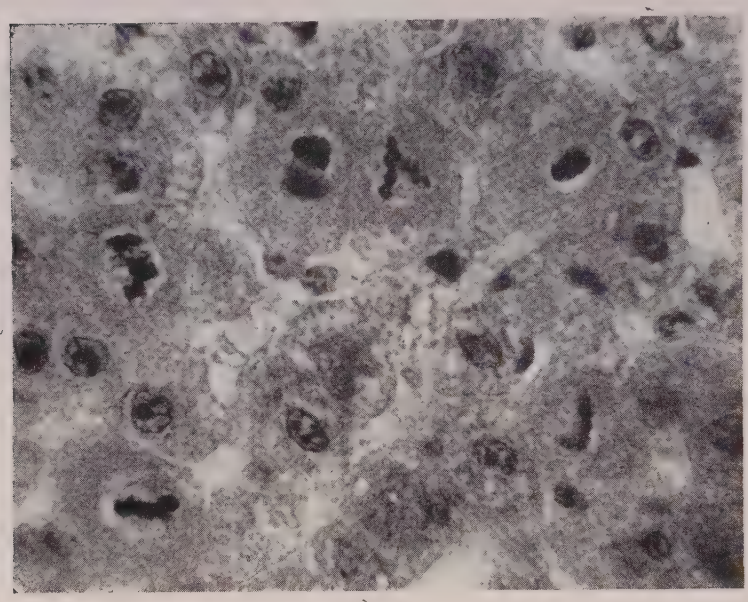

FIG. 1.
Liver of rat treated with 3 doses of 0.4 g thiourea. Mitoses in liver cells. Hematoxylin-eosin. × 850.

mitoses were described by Paschkis and associates.[1]

Mitoses in the normal liver of the adult rat are extremely rare.[2] It is well known, however, that loss of liver tissue by removal of a part of the organ or by necrosis is followed by a compensatory multiplication of liver cells. It was occasionally noted that mitoses in the liver may also occur in the absence of visible changes in the hepatic parenchyma after the administration of staphylococcus toxin,[3] macerated livers, trypaflavine[4] or trypan blue.[5,6] No satisfactory explanation has been advanced for a mitotic reaction of this kind.

The nature of the mitotic response of liver cells to thiourea is likewise not clear. The possibility may be considered that thiourea has a direct stimulating effect on the mitotic activity of the liver cells. On the other hand, occurrence of mitoses may be regarded as a secondary phenomenon, namely as a reparatory reaction following discrete liver damage. The arguments in favor or against each of these possibilities will be dealt with in the complete communication.

Summary. The occurrence of mitosis in the liver cells of rats receiving daily intraperitoneal injections of thiourea is reported. Both mitosis of normal configuration and pathological mitosis were encountered. Thiourea induced mitotic activity of the liver seemed to be at its height during the period of 48 to 72 hours after the first injection.

1 Paschkis, K. E., Cantarow, A., Rakoff, A. E., and Rothenberg, M. S., *Endocrinology*, 1945, **37**, 133.

2 Dawson, A. B., *Growth Suppl.*, 1940, **2**, 91.

3 de Walsche, L., *Arch. de biol.*, Paris, 1931, **42**, 185.

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16016

Role of the Vagus Nerve in Experimental Cinchophen-Ulcer.

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By giving the drug cinchophen, it is possible to produce a peptic ulcer in the dog pathologically identical with that found in man,[1] and, furthermore, procedures which have benefited one have been equally helpful in the other,[2-6] and vice versa.[7,8] Cinchophen produces an ulcer in almost 100% of dogs without grossly disturbing the normal physiology of the gastro-intestinal tract,[9] and provides an ideal means for observing the effect of various proposed measures on peptic ulcer.

Numerous studies on cinchophen-ulcer have been conducted by Stalker, Bollman and Mann.[1,3,5,10] They came to the conclusion that cinchophen produced an increase in the total volume of gastric secretion and that the increase was chiefly acid.[10] Thus, it was assumed that cinchophen caused an ulcer by producing hyperacidity.

If cinchophen-ulcer is caused by an increase in the total volume of acid, it is clear that transthoracic vagotomy, by its reduction of gastric acidity,[11] should have a marked beneficial effect on this ulcer. To verify this inference, transthoracic supradiaphragmatic vagotomy was performed in 15 dogs. These vagotomized animals were then given sufficient cinchophen to produce an ulcer over varying lengths of time.

Procedure. The vagus nerves were observed in the thorax and abdomen at autopsy in 42 dogs and were found to pursue a constant course. Operations were performed under positive intratracheal pressure and morphine-pentobarbital anesthesia. The right chest was opened in the 7th interspace and a 1.5 to 3.0 cm section removed from the anterior and posterior vagus nerves just before they pierced the diaphragm. The chest was closed by interrupted cotton sutures throughout.

Two groups of vagotomized dogs were used. The first, Group A, comprising 4 dogs, received 2 g of cinchophen 4 times a week for at least 30 days (this has been reported as giving the greatest incidence of chronic cinchophen-ulcer[3]) to allow an ulcer to form. Then, 2 g of the drug were given every day to try to perforate the ulcer if present.

The second group, Group B, comprising 11 dogs, received approximately 2 g of cinchophen 6 days a week. This method had been used previously in this laboratory to produce cinchophen-ulcer.[6,7,8]

Results. All the dogs in Group A developed ulcer. Nine dogs in Group B developed ulcer; however, in 2 of these animals the vagi had not been completely severed in the thorax: in one, the posterior branch of the right vagus was intact; in the other, the anterior vagus was intact. The 2 animals in Group B that failed to develop an ulcer received cinchophen for an insignificant period of time—2 to 9 days respectively. All dogs had excellent appetites throughout cinchophen administration until the last day or so before

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TABLE I.
Cinchophen Administration to Vagotomized Animals.

Dog	Total grams of cinchophen	Days	Results
1*	4	2	Stomach and duodenum normal
2*	18	9	" " " "
3*	60	39	4 pyloric antral ulcers
4*	64	32	3 " " "
5*	12	6	Numerous antral ulcers
6*	16	9	3 antral ulcers, 1 fundic ulcer
7*	46	26	3 pyloric antral ulcers
8*	6	4	2 pin-point antral ulcers
9*	18	10	Antral ulcers
10*	74	43	Numerous antral ulcers
11*	50	29	3 large antral ulcers
12†	92	60	Large antral ulcer, large annular pyloric ulcer
13†	114	72	Large annular pyloric ulcer.
14†	118	74	Perforated antral ulcer, healed annular ulcer
15†	113	83	Perforated pyloric ulcer, antral ulcers

* Creighton regime.
† Modified Stalker regime.

death.

Discussion. In dogs, vagotomy produces a decrease in total volume of acidity[11] and also a marked decrease in motility[11,12] which persist for at least 5½ months.[13] Meek[14] reported a peptic ulcer in 2 out of 13 vagotomized dogs but Beazell and Ivy[15] failed to find an ulcer in 60 vagotomized dogs. It is evident, therefore, that vagotomy alone is not capable of producing peptic ulcer in a high percentage of cases. In view of our results, this probably means that merely the increased exposure of the gastric mucosa to gastric contents is not the prime factor in the formation of cinchophen-ulcer in vagotomized dogs.

Since vagotomy decreases the total volume of acidity, it may be that cinchophen produces an ulcer, not by producing an hyperacidity, but by inactivating one of the protective mechanisms of the stomach. Possibly it affects mucus in some way so as to render it incapable of preventing hydrochloric acid and pepsin from attacking the stomach wall. The importance of mucus as a protective mechanism was pointed out by Whitlow[16] and conclusively demonstrated in man[17] merely by allowing a normal amount of acid and pepsin to attack an area of mucosa deprived of mucus.

Summary and Conclusions. Surgical interruption of the vagus nerves above the diaphragm had no effect whatsoever on the incidence of peptic ulcer produced by cinchophen.

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16017 P

Effect of Double Jejunal Lumen Gastrojejunal Anastomosis upon Production of Ulcers by Histamine.

M. E. Steinberg and Milton de V. Brunkow. (Introduced by W. B. Youmans.)

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Von Eiselsberg[1] introduced the exclusion operation for the treatment of duodenal ulcers. The stomach was severed at its distal end. The pyloric segment was closed and the larger proximal segment of the stomach was anastomosed to the jejunum. Devine[2] advocated the division of the stomach at a more proximal level than in the Von Eiselsberg technic. The intent of both of these operations was to exclude the corrosive action of the acid gastric juice from the duodenum and thus bring about the healing of the duodenal ulceration. The results of these operations proved disappointing since jejunal ulcer developed in a majority of the patients operated by the exclusion methods. The Von Eiselsberg and the Devine procedures were tested in the laboratory.[3,4] Jejunal ulcers occurred with regularity when in addition, the alkaline duodenal contents were diverted from the gastrojejunal anastomosis by the Exalto procedure.[5]

The senior author has previously published studies concerning the factor of spasm in the etiology of peptic ulcers.[4,6] In one of these studies a larger jejunal lumen was created by the anastomosis of two parallel jejunal segments in an anti-peristaltic direction.[6] This newly created and enlarged jejunal lumen was anastomosed to the proximal part of the stomach after the closure of the distal segment according to the method of Devine. At the same time the alkaline duodenal contents were diverted from the gastrojejunal anastomosis by the Exalto procedure.[5] None of the 4 dogs operated in this manner and with a double jejunal lumen developed jejunal ulcers. Of the 12 dogs with the Devine and the Exalto operations and with a single lumen gastrojejunal anastomosis, 75% developed jejunal ulcers.[6]

The present studies have been performed for the purpose of determining the effect of this double lumen jejunojejunal anastomosis upon the production of ulcers by histamine-in-beeswax injection. Varco, Code, Walpole and Wangensteen[7] produced peptic ulcers in 100% of dogs with an intact gastrointestinal tract after daily injections of histamine-in-beeswax. Lannin[8] made extensive experiments with the histamine-in-beeswax preparation to determine which of the various gastrojejunal anastomoses gives the most protection against the jejunal ulcer. He was able to produce jejunal ulcers in 100% of dogs subjected to a gastroenterostomy and to the Devine exclusion operation. In our experiments we have performed the gastroenterostomy and the Devine exclusion operation, and in place of the standard single loop gastrojejunal anastomosis we employed the double lumen jejunal anastomosis. Two normal dogs were given daily injections of histamine-in-beeswax, and each of these developed ulcers. Two dogs with double jejunal lumen gastroenterostomy and one with a double jejunal lumen and a Devine exclusion operation were subjected to daily injections of histamine-in-beeswax. One of these 3 animals developed a jejunal ulcer.

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2 Devine, H. B., *Surg., Gynec., and Obst.*, 1925, **40**, 1.

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The other 2 with double lumen anastomosis remained in perfect condition and were sacrificed 43 days after the starting of the injections. No evidence of erosions or ulcers were found in the stomach, duodenum or jejunum.

Protocols. Dog No. 1, weight 30 lb. Operation December, 1946. Gastrojejunostomy with a double lumen jejunojejunal anastomosis. April 7, 1947 histamine beeswax, 30 mg daily. April 29, 1947 dog sacrificed. Shallow round jejunal ulcer, 1 cm in diameter with a pin-point penetration.

Dog No. 3, weight 26 lb. Operation, January 16, 1947. Gastroenterostomy with a double lumen jejunojejunal anastomosis. April 7, 1947, daily injections of 30 mg of histamine beeswax. May 20, 1947 dog sacrificed. No ulcers or erosions found.

Dog No. 4, operation, January 22, 1947. Devine exclusion procedure with a double lumen gastrojejunal anastomosis. April 7, 1947, daily injections of 30 mg of histamine beeswax. May 20, 1947, dog sacrificed. No ulcers or erosions found.

Normal dog No. 1, weight 22 lb. May 12, 1947, daily injections of histamine beeswax. May 20, 1947, dog sacrificed. Shallow ulcers about 1 x 1 cm with induration on the lesser curvature of the pylorus.

Normal dog No. 2, weight 18 lb. May 12, 1947, daily injections of histamine beeswax. April 29, 1947, dog sacrificed. Several small ulcers and erosions in the mucosa of the transverse stomach. Two large ulcers of the first part of the duodenum and one shallow ulcer 4 cm distal to the pylorus.

16018

Effect of Urethane on a Transplantable Acute Lymphoid Leukemia.*

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(With the technical assistance of Lester E. Bunker, Jr.)

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It has been shown that urethane produces a pronounced reduction in blood leukocytes and in immature myeloid cells in both the human and the mouse in cases of myeloid leukemia.[1,2] In addition definite palliative effects have been reported for human beings.[1] The response in human lymphoid leukemias was less pronounced and more variable than in myeloid leukemias. Recently,[3] the response to urethane of spontaneous lymphoid leukemias in the mouse has been studied and the following encouraging effects were obtained: (1) a pronounced fall in blood leukocytes to or below normal levels and maintenance of these levels with continued therapy (2) a marked reduction in the number of immature cells in the circulating blood. (3) a temporary stabilizing effect on hemoglobin levels. (4) a pronounced diminution in size of subcutaneous lymph nodes, spleen and thymus and (5) a significantly greater life expectancy for urethane-treated cases.

A study of the effect of urethane on several acute and chronic lymphoid and myeloid transplantable leukemias in the mouse has been made. This paper summarizes the response of a transplantable acute lymphoid leukemia, line L825, which arose spontaneously in ♀ 79374 (6 months of age) of the C58 inbred leukemic strain of mice. The leukemic

* This work has been aided by grants to the Roscoe B. Jackson Memorial Laboratory by the Jane Coffin Childs Memorial Fund and the National Advisory Cancer Council.

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TABLE I.

Periodic Peripheral Blood Counts of Urethane-Treated and Untreated C58 Mice Inoculated with Acute Lymphoid Leukemia, Line L825.*

Blood counts	7 days					10 days					12 days				
	Hb—g %	Total leucocytes	Lymphocytes	Neutrophils	Lymphoblasts	Hb—g %	Total leucocytes	Lymphocytes	Neutrophils	Lymphoblasts	Hb—g %	Total leucocytes	Lymphocytes	Neutrophils	Lymphoblasts
Urethane†	15.7	5985	45	51	3.6	13.1	6550	30	66	1.6‡	9.1	7970	37	57	4.0‡
Controls	13.6	28950	48	41	10	9.2	76280	23	37	35	5.3	125750	18	20	58

* Means based upon 9 experimental and 9 control animals except at 12-day period where only 2 control animals were alive for blood studies. Same mice used throughout. Blood obtained from tail vein.

† Dosage: 0.75 mg per g body weight per day.

‡ No lymphoblasts found in blood smears of 4 experimental animals.

animals used were of the 32nd, 33rd and 34th transfer generations of this leukemia and were from 5 to 6 weeks of age.

Leukemia line L825 forms a local tumor following subcutaneous transfer and produces massive and diffuse infiltration into the lymph nodes, spleen, liver and thymus in the terminal stages leading to death of mice in 9 to 10 days. The leukocyte count is elevated after 4 to 5 days and lymphoblasts appear at this time. In terminal stages leukocyte counts of 200,000 (90% lymphoblasts) have been encountered.

Urethane was administered daily in the following dosages to 4 separate groups of mice beginning injections 24 to 48 hours after transfer of leukemic cells: 0.75 mg per g body weight, 0.45 mg, 0.25 mg, and 0.15 mg per g body weight. A 6% aqueous solution was given intraperitoneally. Significant palliative effects noted in the experimental series treated with 0.75 mg per g body weight are reported herein.

The blood leukocyte count of urethane-treated leukemics was maintained at levels below normal throughout the period of inoculations of 0.75 mg urethane per g of body weight, whereas the untreated leukemic animals showed a progressive leukocytosis to a mean leukocyte count of 125,750 at 12 days. The absolute lymphoblast count of urethane treated leukemias was 7%, 0.3% and 0.4% of the absolute lymphoblast count of untreated leukemics at 7, 10 and 12 days respectively following transfer of leukemic cells. At 12 days following treatment the mean absolute number of malignant lymphoblasts in urethane treated mice was 318.8 compared with 72935 in untreated leukemics (Table I).

It has been observed in chronic myeloid leukemias in humans that hemoglobin values rise or remain stationary in the majority of cases. No deleterious effects on the red cell series was observed.[1] In our urethane-treated leukemics the hemoglobin levels did not rise nor were they maintained for any length of time. However there were significantly higher values at each blood count period as compared with untreated controls. When the hemoglobin values fell to 5 g %, death ensued shortly

TABLE II.
Effect of Urethane on Infiltration of Leukemic Cells of Lymphoid Leukemia, Line L825, in Various Tissues of C58 Strain Mice.

Exp.	No. animals	Tissue weights in mg/25 g of final body weight*			
		Subcutaneous mass	Spleen	Liver	Subcutaneous lymph nodes‡
Controls	16	1234 ± 93.3†	540.6 ± 41.7	2291.8 ± 158	217.5 ± 13.6
Urethane§	16	230.9 ± 47.6	72.5 ± 6.9	828.8 ± 39.3	62.1 ± 7.3
Difference of means		1003.1 ± 104.7	464.1 ± 42.3	1463 ± 162.7	155.4 ± 15.4
P values		<0.01	<0.01	<0.01	<0.01

* Urethane causes a slight decrease in body weight after many inoculations of sub-anesthetic doses. The means of tissue weights of control and urethane series are weighted equally by using body weights at death of animals.
† Means and standard errors.
‡ Including paired inguinal, axillary, and cervical lymph nodes.
§ Dosage: 0.75 mg per g body weight per day.

thereafter.

Following urethane treatment there resulted a conspicuous decrease in circulating lymphocytes and a corresponding increase in polymorphonuclear leukocytes. This change was evident after 3 daily injections. In view of the fact that this phenomenon occurs in lymphoid as well as in myeloid leukemia the explanation offered for the depression of white blood-cell counts of myeloid leukemias following urethane therapy does not seem tenable.[4]

All urethane-treated mice responded as above described. In 4 mice at the 10 and at the 12 day periods no malignant lymphoblasts could be found in blood smears.

A significantly greater life expectancy was obtained in the urethane-treated (0.75 mg per g daily) series. These experimental animals lived 17.53 ± 0.63 days (25 animals) as compared with 10.37 ± 0.26 days (45 animals) for the controls. The difference of the means of the groups is 7.16 ± 0.69 days where $P<0.01$. On the other hand leukemic mice given smaller doses of urethane did not live longer than untreated leukemics.

A definite retardation of the growth of the subcutaneous mass was evident at 5 days after transplantation of leukemic cells in the urethane series. All urethane-treated mice were negative at this time whereas control leukemic mice showed definite nodules. At 7 days the mean tumor size of urethane-treated animals was 0.98 cm (sum of 2 axes) and of control animals 3.2 cm. There was a profound difference in tumor weights obtained at death of the animals. (Table II). Infiltration into the liver, spleen and subcutaneous lymph nodes as determined by organ weights and by histological study was slight in the urethane-treated series. Urethane-treated mice showed a mean loss in weight of nearly 2 g after 14-15 days of daily injections. It is evident that the loss of weight *per se* in the experimental animals producing some splenic and lymphoid atrophy did not result in the significantly lower organ weights for the following reasons: (1) At 10-12 days following initiation of urethane-therapy, at which time there was no loss in body weight, massive and diffuse infiltration into spleen and subcutaneous lymph nodes was not conspicuous. (2) Organ weights in urethane-treated leukemics were not significantly different from similar organ weights of normal C58 mice of the same age and body weight. (3) It is evident from histological studies that cell destruction of infiltrating lymphoblasts is occurring. Complete histological studies will be given later.

Summary. Urethane, administered intraperitoneally in subanesthetic dose, 0.75 mg per g of body weight per day in leukemic mice transplanted with leukemic cells of the 32nd to 34th transfer generations of an acute lymphoid leukemia, produces the following profound palliative effects: (1) The blood leukocytes are maintained at leukopenic levels. (2) The immature lymphoblasts are main-

[4] Kirchbaum, A., and Lu, C. S., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 62.

tained at significantly lower levels than control leukemics and in some cases disappear entirely from the peripheral blood. (3) Hemoglobin values remain at higher levels in the urethane-treated animals throughout treatment. (4) Local subcutaneous growth of the tumor mass is retarded. Some infiltration of lymphoblasts into spleen, thymus and lymph nodes occurs, however this is not of the characteristic massive and diffuse type. Lymphoblast destruction is evident. (5) A statistically significant greater life expectancy in urethane-treated leukemics is obtained.

16019

Comparison of Rates of Penetration of Unwashed and Washed Spermatozoa in Cervical Mucus.*

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The evidence at hand indicates that the secretion of cervical mucus is under hormonal control.[1-4] It is most abundant when ovulation is believed to occur, *i.e.* at midinterval in the usual 28-day cycle.[5-7] At this time the mucus possesses its lowest viscosity and cellularity,[8] highest water content,[9] and is well supplied with glycogen and other potential reducing substances.[9] *In vitro* experiments show that it is during midcycle that the cervical mucus is most readily penetrable by spermatozoa,[6,8] probably due to the physical and chemical properties that prevail at this time. MacLeod has made the observation that human spermatozoa require glucose or a like monosaccharide for their metabolism and prolonged activity.[10] The fresh semen itself contains some 300 mg per 100 cc of reducing substance expressed as glucose.[11,12] Since spermatozoa probably carry with them little of this extracellular nutriment, they may find in their new environment the necessary substrate for their subsequent metabolism. Enzymes may play an important rôle in the utilization by the spermatozoa of the substances in cervical mucus, but this conjecture constitutes a separate study.

This report is concerned with an endeavor to ascertain the effect of washing of the spermatozoa on their ability to penetrate a column of cervical mucus *in vitro*.

Material and Methods. Subjects: Healthy young women with normal menstrual cycles and pelvic findings supplied the mucus which was collected from the cervical canal by aspiration.[8] Semen specimens were obtained from healthy young donors by manual stimulation. In the evaluation of these specimens consideration was given to the count and

* Aided by a grant from the Ortho Research Foundation, Raritan, N.J.

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12 Huggins, C. B., and Johnson, A. A., *Am. J. Physiol.*, 1933, **103**, 574.

TABLE I.
Rate of Penetration of Unwashed and Washed Spermatozoa in Cervical Mucus.

	No. of specimens	Range of rate of penetration, mm/min	Avg rate of penetration, mm/min
Preovulatory phase			
Unwashed	8	0.0-0.7	0.3
Washed	5	0.0-0.4	0.2
Ovulatory phase			
Ascending			
Unwashed	13	0.6-2.3	1.3
Washed	10	0.5-1.9	1.0
Peak			
Unwashed	6	1.3-2.0	1.7
Washed	10	1.0-2.7	2.0
Descending			
Unwashed	5	0.2-1.8	0.8
Washed	3	0.7-1.4	1.0
Postovulatory phase			
Unwashed	7	0.0-1.5	0.5
Washed	6	0.0-0.6	0.3

morphology.

Preparation of Suspensions of Washed Spermatozoa. One volume of semen and 5 volumes of Ringer's solution were mixed and then centrifuged at approximately 1500 r.p.m. for 10 minutes. The packed spermatozoa were then resuspended in fresh Ringer's solution, the final mixture being brought up to the original volume of semen used. Observations on the counts and morphology were also made on these suspensions. The washing and centrifugation of the spermatozoa resulted in some diminution in the number of motile forms and also in the destruction of some of the cells.

Penetrability. The rate of pentrability was determined by the method of Lamar, Shettles and Delfs.[6] A column of mucus followed by a column of fresh semen or of washed spermatozoa suspended in Ringer's solution was drawn into a capillary tube, leaving a small bubble between the 2 media to serve as a marker. These tubes were then placed on glass slides and covered with mineral oil to reduce refraction for observation under the microscope. By means of a stop watch and a calibrated mechanical stage the rate at which the spermatozoa invaded the cervical mucus was determined.

Results. The mucous cycles were arbitrarily divided into 3 phases: preovulatory, ovulatory, and postovulatory, the ovulatory phase consisting of those days in midcycle during which the amount of mucus was greatly increased and the cellularity decreased. In the normal cycle the amount of mucus increases for 2 to 3 days in the beginning of the ovulatory phase, reaches a peak which is maintained from 1 to 2 days, and then decreases for 2 to 3 days before reaching the postovulatory level.[7,8] Since it has been shown that the rate of penetrability of spermatozoa also follows this general pattern,[6,8] the ovulatory phase was further subdivided into the ascending side, the peak, and the descending side of the curve.

Reference to Table I shows that the rates of penetration of the unwashed spermatoza,[6,8] are in accord with previous observations.[6,8] It can likewise be seen that washing and centrifuging of the spermatozoa did not appear to modify appreciably their ability to invade the mucus. In some tests the spermatozoa, both unwashed and washed, were entirely stopped in their attempt to penetrate the cervical mucus obtained during the pre- and postovulatory phases. However, the average rate of penetration varied from 0.2 to 0.5 mm/min. The highest rate of penetration, *i.e.* from 1.7 to 2.0 mm/min., occurred in those specimens of mucus collected during the ovulatory phase, with some diminution in rate as the collections of mucus departed on either side from the peak.

In these studies measurements of the rate

of penetration were begun promptly after the spermatozoa were brought into contact with the column of mucus. In many cases the spermatozoa on finding the mucus medium particularly favorable, *i.e.* during the ovulatory phase, could be observed to pass through the entire length of the mucus column, an average distance of 25 mm, without apparent deceleration. However, in the pre- and post-ovulatory phases of the cycle when the mucus was more viscid and cellular, the entire distance to which they could penetrate averaged only 1.4 mm. There was no apparent difference between the distances penetrated by the unwashed and the washed spermatozoa.

In all, 42 semen specimens coming from 4 donors were used. These specimens ranged in volume from 0.6 to 4.2 cc and in count from 34 million to 262 million per cc. We were unable, in these studies, to correlate the degree of penetrability of the mucus with the concentration of spermatozoa, either unwashed or washed.

Summary. This study indicates that washing human spermatozoa with Ringer's solution and resuspending the washed spermatozoa in this solution does not impair their ability to penetrate a column of cervical mucus *in vitro*.

16020

Uneven Distribution of Glycogen in the Liver.*

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A strikingly uneven distribution of glycogen in the liver of rabbits was found in the course of experiments in which an attempt was made to follow with biopsies the variation in liver glycogen under various conditions. Irregularities in the intracellular ("glycogen flight") and intralobular[1] distribution of glycogen have been known for some time. Recently, Deane, Nesbett and Hastings[2] have found significant differences in the glycogen content of the two lobes. Since it is obvious that such observations tend to limit the value of liver biopsies in judging the metabolic state of the organ, a short report appears to be warranted.

Experimental. Rabbits were fasted for 18 to 24 hr and laparotomized under sodium pentothal anesthesia. On gross inspection no difference in the appearance of the lobes of the liver was noted. Simultaneous wedge shaped specimens weighing 1000 to 1500 mg were removed from different parts but usually from the same lobe of the liver. Where biopsies were taken at intervals Gelfoam proved very helpful in the control of bleeding. Subsequent biopsies were taken from sites far away from that of the previous resection.

The present report is concerned only with variations observed in simultaneous biopsies. The specimens were cut into 3 portions. The middle slice was fixed promptly in chilled 90% alcohol or Bouin's fluid, embedded, and the sections stained with Best's carmine, Bauer-Feulgen's method[3] and a silver stain.[4] The two lateral portions were weighed and dropped immediately in 30% KOH for the chemical determination of glycogen according to Good, Kramer and Somogyi;[5] the glucose

* This work has beeen aided by a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

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3 Bauer, H., *Z. f. mikr.-anat. Forsch.*, 1933, **33**, 143.

4 Gomori, G., *Am. J. Clin. Path.*, 1946, **10**, 177.

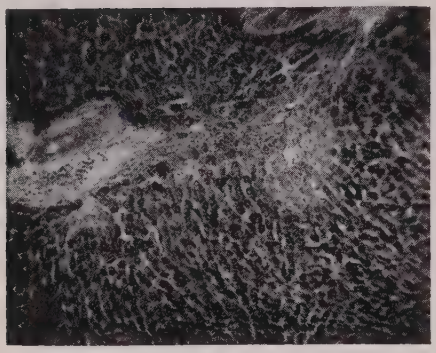

FIG. 1.
Rabbit 52E, biopsy 1. × 80.

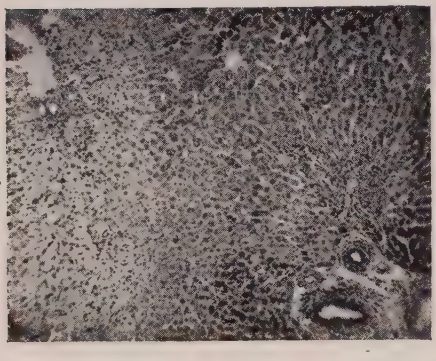

FIG. 2.
Rabbit 52E, biopsy 3. × 80.

formed was determined by the colorimetric method of Folin and Malmros,[6] a photoelectric colorimeter being used.

Table I shows the marked variation in the amount of glycogen found in simultaneous biopsies, the range sometimes exceeding several 100%. The histologic pictures, as far as one can judge from the extent and intensity of the stain, were in good agreement with the

[5] Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, **83**, 115.

[6] Good, A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 485.

FIG. 3.
Rabbit 48, biopsy 3. × 25.
All slides stained with Bauer-Feulgen's stain.

chemical findings, as shown in Fig. 1 and 2. Fig. 3 demonstrates furthermore that even in the very same slide extensive areas of high and low glycogen content may be found side by side.

It should be mentioned that the distribution of fat in the sections, unlike that of glycogen, appeared to be quite uniform.

TABLE I.
Variation in Glycogen Content of Simultaneous Liver Biopsies. (% of wet weight.)

Rabbit No.	Biopsies 1	2	3
48	0.23	0.4	1.6
51 E	0.74	0.9	0.65
52 A	1.6	2.4	—
52 E	1.8	1.5	0.5
53 A	4.2	5.1	—
53 E	2.6	3.5	1.8

Summary. Great caution is warranted in judging the metabolic state of the liver on the basis of the glycogen content of biopsies since variations exceeding several 100% may be observed in simultaneous biopsies taken from the same lobe of the rabbit's liver.

16021 P

Temperature Level and the Growth of Embryo and Tumor of Tumor-Bearing Eggs.

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The technique of growing tumor tissue in embryonated eggs has been routine in this laboratory for more than 4 years. The use of the yolk sac method of inoculation has made it comparatively simple to maintain rat and mouse tumors indefinitely by egg to egg inoculations.[1,2]

The size of the tumors grown in this manner were of the same order as those obtained by transplant in the host animal. This continued to be so after as much as 114 transplant-generations or about 4 years continuously in eggs. Apparently the egg environment satisfies all the requirements of malignant tissue for vigorous growth.

The inoculation of eggs through the yolk sac results in tumor implantation on the yolk sac inner wall.[3] In this situation the growth of the tumor presents a minimum of interference with the development of the chick embryo. Tumor tissue and chick tissue grow together sharing a common blood supply. It is not uncommon for the egg-grown tumor to weigh as much as 6 g at the 17th day of egg incubation, while the chick embyro normally weighing about 20 g at this stage is reduced to 10 or 12 g.

The egg environment is relatively stable but a certain amount of manipulation will be tolerated by the chick embryo. This is especially so with respect to temperature. Incubating eggs will withstand considerable variation in temperature and still survive to the 17-18 day period.

The present paper is concerned with a study of the comparative reactions of tumor and chick tissues grown together to temperatures above and below that required for normal incubation.

Experimental. Embryonated eggs on the 4th day of incubation were inoculated with dba mouse mammary carcinoma. The eggs of one series of inoculations were divided into 3 groups and incubated from the time of implantation until the termination of the experiment at 3 different temperatures. One group representing the control was kept at 99-100° F, a second group was incubated at 96-97°F, and the third group was maintained at a temperature of 103-104°F. At the 18th day of incubation, or 14 days after tumor inoculation, the embryos and tumors of the surviving eggs were harvested and weighed. A total of 1179 eggs was inoculated with tumor tissue for this research involving 17 separate experiments.

The results are given in Table I. Statistical validity of the figures given has been checked and found to be beyond question.

The chick embryo was less affected by the higher- and lower-than-normal incubating temperatures than was the egg-cultivated tumor. This was particularly striking for the temperature range, 103-104°F, at which the chick embryos averaged a reduction in weight of 12% while the tumors grown in these eggs were reduced in size 50% as compared with their respective weights at the control temperature.

Previous work has demonstrated that in eggs inoculated by the yolk sac method the size of the embryo is generally conditioned by the size of the tumor.[4] A large vigorous

1 Taylor, A., Hungate, R. E., and Taylor, D. R., *Cancer Research*, 1943, **3**, 537.

2 Taylor, A., Thacker, J., and Pennington, D., *Science*, 1942, **96**, 342.

3 Hungate, R. E., Taylor, A., and Thompson, R. C., *Cancer Research*, 1944, **4**, 289.

4 Kynette, A., Taylor, A., and Thompson, R. C., *Univ. of Texas Publication No. 4507, Cancer Studies*, 1945, pp. 65-75.

TABLE I.
Effect of Temperatures Above and Below Normal Incubation on Embryo and Tumor Weights of Tumor-Bearing Eggs.

No. exp.	Incubation temperature (°F)	No. eggs inoculated	No. eggs surviving	Avg. chick size (g)	Exp. chicks, control = 100	Avg. tumor size (g)	Exp. tumor, control = 100
17 (control)	99-100	397	165	11.8 ± 1.8	100	1.0 ± .4	100
17	96-97	392	145	10.1 ± 1.2	85.6	.6 ± .2	60
17	103-104	390	55	10.4 ± 1.0	88.1	.5 ± .3	50

tumor frequently occurs in the egg together with an anemic undersized chick embryo. It appears that the relation of the chick embryo to the tumor implanted in its yolk sac is comparable to the relation of the host mouse to a tumor growing from a transplant.

It seems probable therefore that the reduction in size of the egg-grown tumors in response to temperature level above and below 99-100°F was a direct effect. Further, the data indicate that the egg-cultivated tumors are more sensitive to this factor than the supporting embryos.

16022

The Tuberculin Reaction. I. Passive Transfer of Tuberculin Sensitivity with Cells of Tuberculous Guinea Pigs.*†

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(Introduced by Charles A. Evans.)

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It has been established that the "tuberculin type" of sensitivity is distinct from the anaphylactic or Arthus type of sensitivity and from the atopic type of sensitivity dependent upon Prausnitz-Küstner antibody.[1-6] Although it is probable that the tuberculin type of sensitivity depends upon an antibody, the existence of such an antibody has not been established with certainty.

That tuberculin type sensitivity is dependent upon antibody is indicated by the similarities it shows to other sensitivities known to be dependent upon antibody, namely, similarities relating to specificity, incubation time, desensitization, anamnestic reaction, and the correlation between the ability of various animal species to form ordinary antibody and to develop tuberculin sensitivity. Supporting evidence is also provided by the observation that blockage of the reticulendothelial system which depresses the formation of ordinary antibody to an injected protein, likewise suppresses the development of tuberculin sensitivity.[7] In addition, it has been found that the use of adjuvants such as

* This work was supported by a grant from the Alice McDermott Research Foundation of the University of Washington.

† The term sensitivity is used throughout this article in preference to the more commonly used term hypersensitivity.

1 Holst, P. M., *Tubercle*, 1922, **3**, 337.

2 Rich, A. R., Lewis, M. R., *Bull. Johns Hopkins Hosp.*, 1932, **50**, 115.

3 Rich, A. R., *The Pathogenesis of Tuberculosis*, p. 412, Charles Thomas, Springfield, Ill., 1944.

4 Aronson, J. D., *J. Immunol.*, 1933, **25**, 1.

5 Moen, J. K., and Swift, H. F., *J. Exp. Med.*, 1936, **64**, 339.

6 Heilman, D. H., Feldman, W. H., and Mann, F. C., *Am. Rev. Tub.*, 1944, **50**, 344.

petroleum jelly (Petrolatum) and paraffin oil, enhances the ability of heat-killed *Mycobacterium tuberculosis* to engender the development of tuberculin sensitivity[8,9] and, in like manner, of protein antigen to engender ordinary antibody.[10]

Many attempts have been made to accomplish passive transfer of tuberculin sensitivity.[7,11-22] It is notable that in the few reports where success has been claimed the results have been for the most part irregular and difficult to reproduce. In many instances adequate controls were not included or recognition apparently given to the difference between the true tuberculin reaction and the Arthus and anaphylactic reactions.

Helmholz[11] reported successful homologous passive transfer of tuberculin sensitivity with the defibrinated blood of tuberculous guinea pigs.

Bail[12] also reported homologous passive transfer of tuberculin sensitivity by the intraperitoneal injection of ground uncaseated spleen, liver and lymph glands of tuberculous guinea pigs. When the recipient animals were given Old Tuberculin 24 hours later by the intraperitoneal, subcutaneous, and intrapleural routes, most of the animals died within 28 to 31 hours. Bail attributed this to the systemic tuberculin reaction. Control animals given the ground spleen and liver of nontuberculous donors and injected with O.T. in the same manner survived. Cutaneous sensitivity was absent in the recipient animals. However, this is not surprising since the animals were in a very weakened condition and may have been nonspecifically non-reactive.

Joseph,[13] who attempted to repeat Bail's experiments concluded that the results observed by the latter did not prove passive transfer of tuberculin sensitivity. This was based on the observation that the tissue preparations of the donor tuberculous animals were in themselves very toxic. He assumed that the additional toxicity of the tuberculin injected may have been sufficient to kill the recipient animals without the true tuberculin reaction being involved.

Onaka[14,15] confirmed the observations of Bail and of Helmholz but did not agree with the conclusions drawn by the latter.

Massol, Breton and Bruyant[17] reported homologous passive transfer of tuberculin sensitivity with the citrated blood of tuberculous guinea pigs. They employed the technique of reversed passive transfer and used increases in body temperature as an index of tuberculin sensitivity.

Zinsser and Mueller[18] reported irregular success with heterologous passive transfer of tuberculin sensitivity to guinea pigs with the serum of tuberculous rabbits. In successful cases cutaneous reactions could be elicited 3 days following the transfer. However, positive results were infrequent and could not be reproduced at will.

Freund[7] failed to accomplish homologous local passive transfer of tuberculin sensitivity by use of blood serum and organ extracts of tuberculous guinea pigs.

Chase[22] has recently accomplished homologous passive transfer of tuberculin sensitivity in guinea pigs with living cells. The fundamental importance of this finding cannot be overemphasized. It provides the strongest

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7 Freund, J., *J. Immunol.*, 1926, **11**, 383.

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11 Helmholtz, H. F., *Ztsch. Immunitatsforschg.*, 1909, **3**, 371.

12 Bail, O., *ibid.*, 1909, Orig. IV, **1**, 470.

13 Joseph, K., *Beit. z. Klinik der Tuberk. von Brauer*, 1910, **17**, 461.

14 Onaka, M., *Z. Immunitatsforschg.*, 1910, **5**, 264.

15 Onaka, M., *ibid.*, 1910, **7**, 507.

16 Kraus, R., Loewenstein, E., and Volk, R., *Cent. fur Bakt.*, 1911, **1**, 361.

17 Massol, L., Breton, M., and Bruyant, L., *C. R. Soc. de biol.*, 1913, **74**, 185.

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19 Hanks, J. H., *J. Immunol.*, 1935, **28**, 105.

20 Dienes, L., and Schoenheit, E. W., PROC. SOC. EXP. BIOL. AND MED., 1926, **24**, 32.

21 Dienes, L., *J. Immunol.*, 1927, **14**, 43.

22 Chase, M. W., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 134.

evidence, thus far presented, that tuberculin sensitivity depends upon antibody and is not the result of some unknown mechanism capable of simulating antigen-antibody reactions.

Holst[1] observed that tuberculin exerts a toxic action on leucocytes derived from tuberculous animals as evidenced by decreased phagocytic power and loss of differentiation between nucleus and cytoplasm. He clearly established the difference between tuberculin sensitivity and sensitivity due to ordinary antibody by demonstrating that horse serum does not exert an *in vitro* toxic action on the leucocytes of guinea pigs sensitized to that antigen.

Rich and Lewis[2] observed that tuberculin is toxic to the cells of tissue cultures prepared from tuberculin-sensitive animals. Their work was confirmed by Aronson,[23] who noted that the toxic effect of tuberculin on tissue cultures is highly specific.

Aronson[4] made the additional observation that in contrast to this susceptibility of tuberculin-sensitive tissues, tissue cultures from Arthus-sensitive animals are unaffected by specific antigen. This was later confirmed by Rich.[24]

Knowledge of the cytotoxic effect of tuberculin on cultured cells derived from tuberculin-sensitive animals has been greatly extended by the studies of Moen and Swift[5] and Heilman, Feldman and Mann.[6]

The present report is the outcome of some of our initial studies on the nature of the tuberculin reaction. The plan of our investigations necessitated the passive transfer of tuberculin sensitivity. Hence, preliminary trials were made to accomplish passive transfer by the method reported by Chase.[22] In the earliest of these trials, it became apparent that success may be irregular, due in all probability to as yet unrecognized factors.

Experiments. The methods employed were essentially the same as those used by Chase,[22] except that in some of the experiments the donor guinea pigs were subjected to a superimposed infection with the B.C.G. strain of *M. tuberculosis.* The animals were secured from local sources and were of unknown pedigree. They averaged approximately 600 to 800 g in weight. In the first experiments, sensitization of the donors was accomplished by the subcutaneous injection of 25 mg (wet weight) of heat-killed *M. tuberculosis* H37 Rv in paraffin oil. In some of the subsequent experiments, the animals were sensitized by the injection of heat-killed organisms followed several months later by the intraperitoneal injection of 0.5 mg of living B.C.G. and in others by a single intraperitoneal injection of 5 to 10 mg of living B.C.G.

Five to 9 weeks following the sensitizing injections, the animals were skin-tested and the most highly sensitive animals injected intraperitoneally with 30 ml of sterile paraffin oil. Forty-eight hours later, the animals were killed and the cells of the resulting exudate were collected, washed in guinea pig serum-Tyrode solution and injected intraperitoneally into normal mature light-skinned recipient animals. Approximately 90% of the cells were alive as indicated by supravital staining with neutral red and consisted of approximately 10% polymorphonuclear cells, 47% lymphocytes, and 42% large mononuclear cells. Washed cells of the buffy coat of the minced spleens were injected intraperitoneally into other recipients. Each recipient received either the pooled peritoneal or splenic cells of from 2 to 8 donors. The cell volumes transferred to each recipient ranged from 0.4 to 1.0 ml. Control recipient animals received similar preparations from donor controls which had been given a "sensitizing" injection of paraffin oil without tubercle bacilli.

Forty-eight to 72 hours after cell transfer, all animals, including a set of normal controls, were skin-tested with O.T. in dilutions ranging from 1:10 to 1:100 or with deglycerinated O.T. employing the lowest dilution which failed to give a reaction in normal animals.

A total of 48 donors and 18 recipients were used in the various experiments. The results of the first experiment, in which donors sensitized with heat-killed tubercle bacilli were

[23] Aronson, J., *J. Exp. Med.*, 1931, **54**, 387.

[24] Rich, A. R., *The Pathogenesis of Tuberculosis*, p. 409, Charles Thomas, Springfield, Ill., 1944.

used, were negative except for a slight reaction in one recipient. The reaction in this animal became apparent after 12 hours and reached its height by 24 hours at which time it presented a 15 mm area of 2+ erythema and edema.

A careful consideration of this trial led us to believe that the most probable cause for our lack of success was that the strain of donor animals we used possessed a low ability for developing sensitivity. They showed 4+ reactions to 1:100 O.T. but only 3+ reactions to 1:100 O.T.

In an effort to render the donors more sensitive we next sensitized the animals with either heat-killed *M. tuberculosis* H37Rv and living B.C.G. or living B.C.G. alone, as outlined above. The majority of these animals developed a strong sensitivity. They reacted to 1:1000 O.T. with extensive necrosis and gave positive reactions to deglycerinated O.T. with dilutions of 1:40,000 and higher. The yield of peritoneal cells from these donors was about double that of the donors sensitized with heat-killed tubercle bacilli. Most of the recipients that received cells from these animals gave positive reactions to tuberculin. The reactions in animals sensitized with peritoneal cells were strong and of a typical tuberculin type. They were commonly negative for the first 10 hours, became fully developed by about the 30th hour and consisted of 15 mm areas of 3+ erythema, edema and induration, with a sharp central 5 mm zone of ischemic blanching. The reactions usually began to fade by the 40th to 50th hour, but were still apparent at 72 hours or longer. In several instances the early reactions were so intense that necrosis was anticipated but never developed. Intense reactions were elicited with dilution of O.T. as high as 1:100.

The animals that received splenic cells were usually less sensitive. They gave typical but less intense tuberculin reactions which lacked central blanching and faded earlier. The skin tests of most of the control animals were negative. The few controls which reacted presented small areas of 1 + erythema and edema which clearly differed from the reactions of the test animals by fading earlier and being devoid of central blanching. Fading was evident by the 24th hour and complete by the 48th hour.

The transferred cutaneous sensitivity was of short duration. Skin tests made 5 days after cell transfer were negative.

A preliminary attempt was made to elicit the systemic tuberculin reaction in two of the recipient animals by the intraperitoneal injection of 2 ml and 0.6 ml of O.T. respectively. The material was prepared for injection by dilution with several volumes of physiological saline. The animals reacted with a delayed type of shock which came on after about 3 hours. They were in severe shock by the 8th hour. The animal that received 2 ml of O.T. was dead at 24 hours and the other animal recovered. A second similar dose of O.T. administered to this animal 24 hours after the first was without effect. Normal control animals that were similarly injected remained unaffected.

Discussion. As Chase has emphasized, the successful passive transfer of tuberculin sensitivity in the guinea pig apparently depends upon the transfer of a large number of living leucocytes from highly sensitive donor animals.

A question of much interest and importance is whether an antibody is involved and what its nature may be. If an antibody is responsible for reactions to tuberculin, it is important to explain the apparent sessile existence of this "tuberculin antibody." Perhaps one of its properties is that it is avidly absorbed by tissues. This could account for its wide distribution and apparent sessile existence since the quantity of antibody in the circulation may thus be kept at such low levels as to be undetectable. A less-likely alternative is that all cells exhibiting sensitivity are capable of forming the antibody which remains fixed in the cells.

There appears to be little doubt that the transfer accomplished in the present experiments was passive and not active because of the short incubation period and transient nature of the sensitivity. In fact, the duration of tuberculin sensitivity for only a few days is so much shorter than the Arthus, anaphy-

lactic and Prausnitz-Kustner sensitivities as to indicate that a different and perhaps quite labile antibody must be concerned. The fact that passive transfer of tuberculin sensitivity has been accomplished only with living cells and only after an incubation period of two to three days suggests that the sensitivity may be principally due to antibody elaborated by such cells during their residence in the recipient rather than to preformed antibody.

Summary. Homologous passive transfer of tuberculin sensitivity was accomplished with the peritoneal and splenic cells of guinea pigs infected with the B.C.G. strain of *Mycobacterium tuberculosis.*

The possible mechanism and factors involved in passive transfer of tuberculin sensitivity are discussed.

16023

Effects of X-ray Irradiation on Viscosity of Synovial Fluid.

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(Introduced by R. F. Loeb.)

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The treatment of arthritis by X-rays advocated in the European[1] and English[2] literature since 1930 is difficult to evaluate. However, in 1941, Smyth, Freyberg and Peck[3] demonstrated conclusively that a favorable response to X-ray therapy is obtained in ankylosing spondylitis. Many confirmatory reports have followed this observation.[4] The mechanism whereby improvement follows roentgenotherapy in such cases is not apparent.

Recently, two papers[5,6] have appeared describing a reduction in the viscosity of thymonucleic acid after irradiation, depending in degree upon the total X-ray dosage given. This is in agreement with the principles outlined by Colwell[7] in his review of work dating from 1912, to the effect that a diminution in viscosity of organic colloids such as egg-white, serum and starch followed exposure to X-ray. The reason such a change occurs is still not clear. In the case of thymonucleic acid,[6] evidence was presented that the change induced by X-radiation did not involve a splitting of primary linkages or a rearrangement of the configuration which made it susceptible to enzymatic attack.

The viscosity of normal human synovial fluid is very high.[8,9] The viscosity of joint fluids obtained from the knees of patients with rheumatoid arthritis tends to be greater in the chronic than in the acute stage, and to approach the viscosity of normal synovial fluid.[10]

The present study records observations which show that irradiation of joint fluids

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4 Kuhns, J. G., and Morrison, S. L., *N. E. J. Med.,* 1946, **235**, 399.

5 Sparrow, A. H., and Rosenfeld, F. M., *Science,* 1946, **104**, 245.

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8 Schneider, J., *Biochem. Z.,* 1925, **160**, 325.

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10 Unpublished observation.

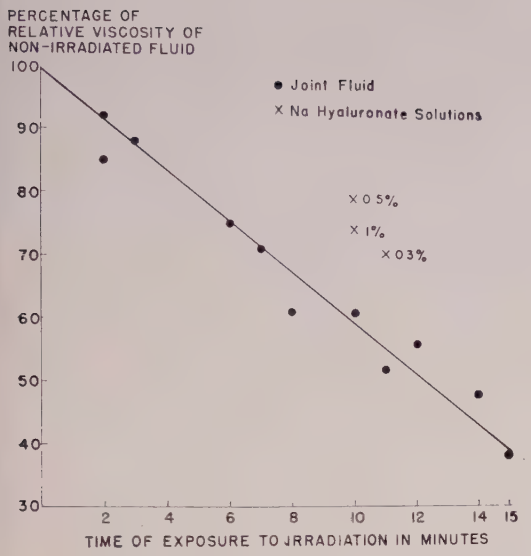

FIG. 1.

Effect of X-ray irradiation on viscosity of joint fluid and Na hyaluronate solutions.

diminishes their viscosity and that the decrease bears a direct relationship to the amount of irradiation.

Methods. Relative viscosity was measured in 5 cc Ostwald viscosimeters in a water bath at 21±1°C. Total protein concentrations of the synovial fluid were determined with a gradient tube method.[9,11] The amount of hyaluronic acid present was measured by a turbidimetric method[12] similar to that described by Seastone.[13] Irradiation was furnished by a Phillips contact machine delivering 9290 r/minute. Material to be irradiated was placed in a 50 cc glass centrifuge tube in contact with the treatment tube of the X-ray machine. The average roentgen delivery to the fluid was 334 r/minute.* Because of the type of irradiation, this estimate is only approximate.

The results of the irradiation of 4 pathological knee joint fluids† are shown in Fig. 1. Their relative viscosities were 214, 59.4, 42.7 and 22.5 x H_2O. When these were plotted against time of exposure, the correlation between the time of irradiation and the percentage decrease in viscosity was good.

The effect of irradiation on solutions of pure sodium hyaluronate‡ of varying concentrations was less than the effect on joint fluid (Fig. I.). Upon irradiation of the pure solutions, no increase in titratable acidity above that of the unirradiated controls was demonstrable, which would imply that there was no oxidative degradation. In irradiated joint fluids, no appreciable decrease in the amount of hyaluronate was detectable turbidimetrically nor was there any change in the total protein content of the fluid (Table I.). A small difference in the enzymatic hydrolysis of the irradiated and control samples was detectable viscosimetrically. The half-time for the irradiated sample was 14.5 minutes, and of the control 17 minutes, when equal amounts (0.05 mg equal to 7 viscosity reducing units) of bull testis hyaluronidase were used. From this one may assume that no profound change in the hyaluronic acid molecule had occurred.

An effort was made to detect the continuing fall in viscosity on standing after irradiation, as described by Taylor *et al.*[6] for thymonucleic acid. Some indication of this was seen in the preparations of pure hyaluronate, but not in the joint fluid preparations.

Discussion. Irradiation causes a decrease in viscosity of joint fluid. It was impossible to determine the exact amount of irradiation delivered under our conditions, but the decrease bore a direct relation to the time of exposure. The manner in which the molecule is changed is not clear from these observations. That purified hyaluronic acid is

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* This was calculated with an ionization chamber in the fluid at 3 sites: 1. Nearest the X-ray tube. 2. In the center of the centrifuge tube. 3. At the side the greatest distance from the X-ray tube. The results of the three readings were averaged.

† Two of the fluids were obtained at different times from a patient with rheumatoid arthritis. One was obtained from a patient with an undiagnosed arthritis of the knees, possibly associated with lymphogranuloma inguinale. One was a pooled sample containing knee joint fluid from several patients with rheumatoid arthritis.

‡ Obtained from Dr. K. Meyer.

TABLE 1.
Effect of X-ray Irradiation on Relative Viscosity, Hyaluronic Acid Concentration, and Total Protein Content of Joint Fluid.

Joint fluid	Time exposed to irradiation (min)	Relative viscosity X H_2O	Total protein, %	Hyaluronic acid (mg per cc)
R.R. of 6/18/46	0	214	4.8	0.24
	2	200	4.9	0.26
	7	151	4.9	0.25
	11	111	4.9	0.24
Pooled	0	22.5	4.9	0.22
	2	19.2	4.9	0.21
	6	16.9	4.9	0.22
	12	12.7	4.9	0.22
R.R. of 3/12/47	0	42.7	5.0	0.16
	3	37.4	5.0	0.16
	8	26.1	5.0	0.16
	14	20.5	5.0	0.16

never as viscous as joint fluid of equal hyaluronic acid concentration[12] has been attributed to the presence of secondary valences in the native material which are broken on purification. The greater susceptibility to irradiation of joint fluid, as compared with that of solutions of hyaluronate, would suggest that these secondary valences may primarily be attacked by the X-rays. The hyaluronic acid in irradiated joint fluid is still susceptible to enzymatic hydrolysis and thus irradiation did not cause a profound change in the hyaluronic acid molecule. Although the amount of irradiation delivered is beyond the physiological range, it is possible, nevertheless, that this effect may be one of the factors which come into play in the response of pathological joints—peripheral and spinal—to irradiation. The widespread presence of the viscous acid mucopolysaccharides of this group throughout the connective tissue of the organism may make this an important factor in the response of many tissues to roentgenotherapy.

Conclusions. 1. The viscosity of joint fluid is decreased by exposure to X-rays, the decrease in viscosity being directly proportional to the amount of irradiation. 2. The viscosity of pure hyaluronic acid solutions is decreased less by the action of X-rays than is the viscosity of joint fluid. 3. Joint fluid is as susceptible to the enzymatic action of bull testis hyaluronidase in the irradiated sample as in the control. No increase in titratable acidity follows irradiation of pure hyaluronate solution, indicating that the reduction in viscosity probably does not result from oxidative degradation. 4. The possible physiological implications are discussed.

16024

Lack of Effect of Secondary Liver Extract (No. 55) on Absorption of Radioactive Iron.*

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Introduction. It has long been recognized that in anemias due to chronic hemorrage, either experimental or pathological, iron is efficient in the restoration of the normal blood picture. An inability on the part of the body to form enough of the porphyrin fraction of the hemoglobin molecule has never been established, even under the most stringent conditions. It is with extreme difficulty[1] that one can demonstrate the limitation of hemoglobin production under experimental conditions through the lack of protein for the formation of the globin part of hemoglobin. That other factors are involved, however, in the production of hemoglobin or the stimulus to its production, is suggested by the fact that Whipple, Robscheit-Robbins, and Walden were able to show[2] that a liver fraction other than that most active in the treatment of pernicious anemia was capable of causing new hemoglobin formation. That the effect was not due to the iron content alone was suggested by the observation that there seemed to be "summation response" when this extract and iron were given in combination as contrasted to the administration of either alone.

This liver fraction, called the secondary anemia fraction, or No. 55 fraction, contains that part of the liver material insoluble in 70% alcohol as distinguished from the "pernicious anemia fraction" (No. 343 fraction).

It seemed of interest to determine whether or not there was a possibility that such a fraction might be involved in the augmentation of iron uptake from the gastro-intestinal tract. With the availability of the sensitive means of determining uptake, using iron tagged with the radioactive isotope Fe^{59}, one is provided with a tool with which such an effect could easily be tested. That this "summation effect" is due to an increase in the absorption of iron from the gastro-intestinal tract seems to be pretty well ruled out by the following experiments.

Methods. The subjects studied were 11 normal women, laboratory technicians or secretaries, ranging in age from 21 to 45 years. In addition, 1 patient, who had had repeated massive hemorrhage due to duodenal and gastric ulcers, was included. The subjects were divided into 2 groups, half receiving at the first feeding iron tagged with the radioactive isotope alone and the other half receiving the same dosage level of tagged iron, plus 5 g of liver extract No. 55.† Two weeks after the feeding a single sample of whole blood was drawn into ammonium and potassium oxalate, and this was divided into duplicate samples for estimation of radioactivity content of the red blood cells. At this time those subjects who had received iron alone were then given the same dosage of iron plus the liver extract, and that group which had received both iron and extract were given iron alone. Two weeks later the blood was sampled again, and feeding reverted to the original program as of the first experimental period. In this way each subject acted as his own control.

The radioactive iron was purified as described elsewhere[3] in order to eliminate contamination due to traces of radioactive cobalt, manganese, zinc, nickel, copper, indium, etc. The iron isotope used was a cyclotron

* This work was carried out under a grant from the Nutrition Foundation.

1 Hahn, P. F., and Whipple, G. H., *J. Exp. Med.*, 1939, **69**, 315.

2 Whipple, G. H., Robscheit-Robbins, F. S., and Walden, G. B., *Am. J. Med. Sci.*, 1930, **179**, 628.

† The No. 55 Liver Extract fraction was provided through the courtesy of Dr. W. W. Davis of the Eli Lilly Company.

3 Hahn, P. F., *Ind. and Eng. Chem.*, 1945, **17**, 45.

TABLE I
Effect of Concomitant Feeding of Secondary Liver Extract (No. 55 Fraction) on Absorption of Iron Tagged with the Radioactive Isotope Fe^{59}. Dose of Fe = 39 mg.*

Subject	Wt kgm	RBC Hct.%	Uptake of tagged iron in % of administered dose when fed			
			Fe Alone	Fe + Liver	Fe Alone	Fe + Liver
E. C.	48.6	43	9.1	7.8	7.9	
P. J.	48.2	42		4.1	6.4	0.7
B. R.	53.5	44	1.4	1.4	3.9	
M. C.	50.4	44		5.3	9.9	7.1
D. R.	49.2	38	5.0	3.0	3.7	
J. C.	44.6	41		2.1	1.7	1.2
I. L.	39.7	43	5.1	1.2	4.2	
J. A.	55.8	44		1.5	7.4	2.3
M. G.	58.9	41	3.6	1.1	3.6	
B. P.	57.6	43		8.9	9.8	6.8
H. T.	52.2	42			4.7	7.8
C. P.	73.6	22	49.0	47.0		

* Except Subject C.P. who received 25 mg.

prepared product, Fe^{59}, made by the d-p reaction on Fe^{58}. The blood withdrawn was centrifuged in graduated 15 ml tubes in a type 1 International standard 8 unit head for 35 minutes at 2800 r.p.m. or more. The plasma was discarded following reading of the hematocrit value, and the red cells washed into Pyrex beakers, dried, ashed in a muffle furnace at 625°C, and the resulting iron electroplated as described elsewhere.[3] Radioactivity measurements were made, using a thin mica window, argon filled bell type Geiger counting tube, in conjunction with M.I.T. counting rate meter. The assumption was made that all iron absorbed was utilized, and the calculation of uptake was based on calculations described previously.[4]

Experimental Observations. In Table I, below, are summarized the results of the experiments carried out. The iron uptakes as measured with the radioactive tracer varied in these women from 1½ to 10% of the administered dose. The series involved is too small to allow one to speculate effectively on the meaning of such variation. It is to be noted, however, that with such a wide range of uptake, one must interpret carefully the absorption of single doses of iron in attempting to use this technique for diagnostic purposes.[3] It was for this reason that the procedure was carried out as described above, where each individual would furnish a control for each particular experiment. It is obvious on inspection of Table I that there is no enhancement of the uptake of the tagged iron resulting from the concomitant administration of the secondary liver extract. If anything, there is a suggestion that the liver extract might conceivably, to a small extent, inhibit the absorption of iron, although this may not be statistically significant. The rationale of such an inhibition might be related to the presence of phosphates or related material, which act to precipitate iron in the gastro-intestinal tract and prevent its absorption before it has passed beyond the stomach and duodenum.

Discussion. It is of interest to note that the one individual who had a marked iron deficiency type of anemia related to repeated acute and chronic blood loss, patient C.P. as shown in the table, showed the typical high efficiency of absorption and utilization of iron which one associates with such a condition, and this contrasts very markedly with the uptakes in the normal laboratory workers and secretaries who made up the remainder of the experimental group of subjects.

It is necessary to point out that these experiments, in spite of the sensitivity of quantitation attainable through the use of the radioactive isotopes over the technique used by Whipple and Robscheit-Robbins, differed

4 Hahn, P. F., Bale, W. F., Ross, J. F., Balfour, W. M., and Whipple, G. H., *J. Exp. Med.*, 1943, **78**, 169.

in one possibly important matter in that they represented the feeding of single small doses of tagged iron; whereas, the work done on dogs by the other investigators represented daily feedings of iron and liver extract over several weeks periods. Recently Granick[5] has shown that the ferritin of the gastro-intestinal tract increases following the feeding of iron. Thus we must keep in mind the possibility that the body may adapt itself to the absorption of larger amounts of iron through some as yet unknown mechanism for the production of the material which itself is probably concerned with iron absorption.

It is important also to keep in mind the fact that the experimental standard anemic dogs used by Whipple and Robscheit-Robbins in their classical experiments on factors involved in hemoglobin regeneration are not simple "iron deficiency" experimental animals. The iron content of the salmon-bread diet fed these animals is adequate for normal growth and maintenance, but probably not sufficient to provide for the increased demands imposed by the necessity of removal of large amounts of blood occasioned by feedings of active supplements. Since it has been shown, as mentioned earlier,[1] that under certain circumstances restriction of proteins may cause a lowered ability to form sufficient hemoglobin in experimental hemorrhagic anemia in dogs, it is possible that under the conditions of the experiments cited by Whipple, Robscheit-Robbins, and Walden that there was a more complicated deficiency involved, in which more than one factor or two interrelating factors were involved.

Whatever the explanation may be as to the different results obtained under these widely various sets of conditions, it is apparent that any influence on hematopoiesis exerted by liver extract No. 55 is not one which acts through its ability to augment the absorption of the iron from the gastro-intestinal tract.

Summary. Iron tagged with the radioactive isotope Fe^{59} was fed with and without supplements of liver extract No. 55 to a group of 11 normal adult women. There were no obvious differences in the absorption and utilization of iron when this material was given alone or in conjunction with the "secondary anemia fraction of liver." In one case of human iron deficiency due to multiple acute and chronic hemorrhage there was also no difference in the absorption of iron as indicated by this method.

The "summation response" described by Whipple, Robscheit-Robbins and Walden is apparently not related to the effect of the secondary liver extract on *absorption of iron* in the gastro-intestinal tract.

[5] Granick, S., *J. Biol. Chem.*, 1946, **164**, 737.

16025

The Influence of Oral Saccharin on Blood Sugar.

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The purpose of this investigation was to determine whether or not sweet taste as a nervous sensation has an effect on carbohydrate metabolism. It was found that saccharin, a substance of intensely sweet taste, can cause a decrease in blood sugar, presumably through the reflex liberation of insulin.

Methods. Saccharin (Sodium salt o-benzoic-acid-sulfimide) was given to human subjects in doses of 0.05 g in 80 ml water. In one group of experiments the effect of single doses, and in another group the effect of repeated doses of saccharin solution was followed.

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TABLE I

Blood sugar values, determined at 10 minute intervals in individuals under normal fasting condition (F) and in the same individuals under the same conditions after oral administration of 0.05 gm saccharin in 80 ml water (S). Blood sugar values are expressed in terms of percentage of the blood sugar level at 0 time.

Indiv. Subj. No.	10 min		20 min		30 min		40 min		50 min	
	F	S	F	S	F	S	F	S	F	S
1.	96.2	98.4	104.2	90.0	98.1	84.3	102.1	93.4	98.8	90.1
2.	98.1	100.1	102.4	91.4	97.3	85.2	96.4	87.6	96.2	98.3
3.	97.0	96.2	100.1	93.2	96.4	88.7	100.1	88.2	103.2	104.1
4.	96.6	98.8	98.4	85.1	101.1	90.3	95.4	95.1	99.3	99.1
5.	101.4	99.3	97.3	80.3	95.2	80.2	98.3	85.3	100.1	97.5
6.	97.6	102.4	96.4	90.1	93.4	78.1	97.6	81.1	98.8	93.6
7.	104.2	101.3	101.3	83.1	95.3	84.1	99.4	92.1	92.3	97.2
8.	98.9	97.7	100.2	92.2	96.0	85.3	96.2	98.6	100.0	106.9
9.	100.1	99.8	97.0	95.4	97.1	82.0	97.0	99.1	95.4	94.2
	M=98.8	M=99.3	M=99.6	M=88.8	M=96.6	M=84.2	M=98.0	M=91.2	M=98.2	M=97.9
	t = 0.5		t = 5.7		t = 7.7		t = 3.2		t = 0.15	

In the first group 9 persons were used. In the morning, before eating, the subjects remained in bed, and blood sugar determinations were carried out at 10 minute intervals for 50 minutes. Blood was taken from the cubital vein and blood sugar determinations were done with 0.1 ml samples by an iodometric titration method.[1] A fasting level was thus established. Each subject was then used at the same time the next day under the same conditions for the saccharin experiment. Ten minutes after the first blood sample was taken, the subject drank a solution of saccharin (0.05 g dissolved in 80 ml of water). As standard procedure the drinking lasted for 5 minutes.

Results. In 5 control experiments 80 ml water had no significant effect on the blood sugar. The results are given in tables where the blood sugar values are expressed in terms of per cent of the initial blood sugar level. This initial level was arbitrarily taken as 100. In Table I are shown the changes in blood sugar level following a single saccharin administration. The means of 9 parallel experiments were compared with the means of 9 controls. The significance of the difference between the means was tested by calculating the "t" values according to Fisher.[2] Following administration of the saccharin solution a rapid decrease in blood sugar occurred which ended after 30 minutes.

The second group of 6 persons was given the same amount of saccharin solution as was given to Group I, but it was divided into 4 parts which were drunk at 10 minute intervals. Blood samples were drawn at 15 minute intervals for 90 minutes. The administration of saccharin solution in 4 parts resulted in the same drop in blood sugar, except that the hypoglycemia lasted 75 minutes. The results of this experiment are shown in Table II.

Discussion. A comparison of the 2 series of experiments reveals no quantitative correlation between the decrease in blood sugar and the amount of saccharin taken. Salkowski,[3] and Carlson, Eldridge and Foran[4] showed that saccharin has no pharmacological activity in the small doses used here. In both experiments, the sensation of intense sweet taste was produced and there is good reason to believe that this taste, and not the amount of saccharin was responsible for the blood sugar effect. It would be of interest to test other sweet tasting substances for this effect. Syllaba[5] reported an increase in blood sugar after the administration of saccharin by means of a gastric tube in rabbits and man. He suggested that this effect is due to a reflex mechanism between the intestinal wall

[1] Fujita, A., and Iwatake, D., *Biochem. Z.*, 1931, **242**, 43.

[2] Fisher, R. A., *Statistical Methods for Research Workers*, 1930, Oliver and Boyd, London.

[3] Salkowski, A., *Virchows Arch.*, 1886, **105**, 46.

[4] Carlson, J., Eldridge, and Foran, *J. Metabol. Res.*, 1929, **3**, 451.

[5] Syllaba, G., *Am. J. Physiol.*, 1929, **90**, 535.

TABLE II

Blood sugar values determined at 15 minute intervals in individuals under normal fasting conditions (F) and in the same individuals under the same conditions after oral administration of 0.05 gm saccharin in 80 ml water, given in 4 equally divided doses at 10 minute intervals after 0 time. Blood sugar values are expressed in the same way as in Table I.

Indiv. Subj. No.	15 min		30 min		45 min		60 min		75 min		90 min		105 min	
	F	S	F	S	F	S	F	S	F	S	F	S	F	S
1.	98.0	100.0	101.2	87.2	100.0	81.2	96.2	91.0	95.4	93.6	99.3	106.1	98.3	104.2
2.	96.0	86.0	104.4	84.3	96.2	84.0	95.3	90.2	98.0	85.4	105.3	97.6	105.1	102.1
3.	101.0	88.0	96.0	86.2	98.5	85.7	101.0	81.6	96.2	90.0	103.7	92.1	102.0	106.1
4.	97.0	97.1	100.0	89.2	102.2	82.0	103.0	83.7	101.0	87.3	96.7	106.0	100.1	104.2
5.	98.0	90.7	97.4	87.0	97.1	86.3	102.7	80.0	97.3	91.1	102.0	95.5	97.3	97.3
6.	99.2	90.6	98.8	84.0	99.0	82.5	100.4	85.3	98.0	88.3	104.1	106.1	99.3	96.2
	M=	M=	M=	M=	M=	M=	M=	M=	M=	M=	M=	M=	M=	M=
	98.3	91.0	99.6	86.3	98.8	83.5	99.6	84.0	97.7	89.2	101.8	100.5	100.3	100.1
	t = 5.6		t = 7.7		t = 10.4		t = 6.5		t = 6.0		t = 0.4		t = 0.1	

and the liver. Althausen and Wewer[6] could not confirm this observation. These authors disregarded the relatively small but reproducible decrease in blood sugar after saccharin consumption, although according to their results this decrease was observed. Fischer and Schroter[7] investigated the effect of saccharin on human patients suffering from various diseases, but no control tests on healthy subjects were carried out. Some of these patients showed a decrease in blood sugar, but the authors attached no significance to this phenomenon. The small but significant decrease in blood sugar level can be seen only under circumstances in which all other influences are eliminated or controlled in every way possible. As an explanation of the observed phenomenon, it seems logical to think of insulin liberation, called forth by a nervous impulse through the sensory fibers from the taste endplates to the vagus centers. Since Geiger's experiments[8] showed that the vagus is a secretory nerve of the islets of Langerhans, it is possible that the efferent part of this reflex arc is in the vagus. Other afferent stimuli from sensory organs, such as the olfactory, cause a similar decrease in blood sugar, as reported by Bassi and Pascucci,[9] whose observation also strengthens the possibility of the existence of the above described reflex mechanism.

Summary. 1. Oral administration of an aqueous solution containing 0.05 g of saccharin caused a decrease of 12 to 16% in the blood sugar level of normal individuals. Water alone had no significant influence.

2. The decrease of blood sugar was not proportional to the saccharin dosage.

3. Administration of the saccharin in 4 portions during a 40 minute period increased the duration of hypoglycemia.

4. It is suggested that this phenomenon is due to the influence of the sweet taste, which may act by means of a reflex mechanism to induce insulin secretion.

6 Althausen, T. L., and Wewer, G. K., PROC. SOC. EXP. BIOL. AND MED., 1937, **35**, 517.

7 Fischer, F., and Schroter, A., *Dtsch. Med. Wschr.*, 1935, **61**, 1354.

8 Geiger, E., *Arch. Exp. Path. and Pharmacol.*, 1928, **134**, 317.

9 Bassi, M., and Pascucci, P., *Rass. Neur. Veget.*, 1943, **3**, 68.

16026

Action of Sodium Salicylate on Prothrombin Time in Rabbits.*

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Link[1] has postulated, on the basis of the degradation of dicumarol to salicylic acid *in vitro*, that the hypoprothrombinemic action of dicumarol is due to its degradation to salicylic acid *in vivo*. Supporting this hypothesis was the finding of Link, Overman, Sullivan, Huebner, and Scheel[2] that single doses of salicylic acid administered either orally or intravenously to rats maintained on an artificial diet low in vitamin K, caused a temporary hypoprothrombinemia. The increase in prothrombin time after administration of salicylates has been confirmed clinically by Rapoport, Wing and Guest,[3] Shapiro[4] and others. However, Link's views on the relationship of the action of dicumarol and salicylate on prothrombin have not been supported. Link's data indicate that salicylates have a very weak action in lowering prothrombin compared with dicumarol. Lester[5] failed to find salicylates in the urine after the administration of dicumarol to rats. Stefanini and Petrillo[6] observed that the addition of sodium salicylate to human plasma *in vitro* in concentrations greater than 0.2% markedly lengthened the prothrombin time. Clark and Spitalny[7] have observed that other analgesic-antipyretic drugs (antipyrine, aminopyrine, acetanilid, acetophenetidin and cinchophen) have a similar action on prothrombin time, and that the prothrombinopenic action of salicylate was greatly augmented by hyperthermia and by an increased metabolism from other causes. This suggests that an important contributing factor to the action of salicylates on prothrombin clinically is the accompanying clinical syndrome, and that the effect observed by Link and co-workers is a non-specific effect, unrelated to the action of dicumarol.

Since dicumarol has been synthesized from salicylic acid and since dicumarol is presumably formed from the coumarins in spoiled sweet clover by bacterial action,[1] there remains as a further possible basis for the prothrombopenic action of salicylates, the conversion of these substances to dicumarol or related compounds. To test this possibility, a study has been made of the response to dicumarol and sodium salicylate in rabbits upon oral and intravenous administration.

Methods. Normal rabbits of 2 kg body weight and maintained on the normal colony diet were used. Prothrombin times were determined daily, on both undiluted plasma and also on 50, 25, 12.5 and 6.25% plasma. As reported by previous workers, the salicylate had little effect on the prothrombin time of undiluted plasma so that all the results reported are for the 12.5% plasma. Prothrombin times were determined by the Quick technique, using an acetone-dried horse brain powder for preparation of the thromboplastin.[8] We are indebted to Dr. L. A. Kazal of Sharp and Dohme, Inc., who kindly supplied this material. To control minor variations in thromboplastin, in all cases a normal rabbit with the same initial prothrombin time was included in each series and the prothrombin times are reported as experimental/(normal

* Aided by a grant from the National Research Council of Canada.

1 Link, K. P., *Harvey Lecture Series*, 1943-4, **39**, 162.

2 Link, K. P., Overman, R. S., Sullivan, W. R., Huebner, C. F., and Scheel, L. D., *J. Biol. Chem.*, 1943, **147**, 463.

3 Rapoport, S., Wing, M., and Guest, G., Proc. Soc. Exp. Biol. and Med., 1943, **53**, 40.

4 Shapiro, S., *J. Am. Med. Assn.*, 1944, **125**, 546.

5 Lester, D., *J. Biol. Chem.*, 1944, **154**, 395.

6 Stefanini, M., and Petrillo, E., *Boll. Soc. Ital. Biol. Sper.*, 1946, **22**, 366.

7 Clark, B. B., and Spitalny, M., *Fed. Proc.*, 1946, **5**, 171.

8 Kazal, L. A., Higashi, A., and Arnow, L. E., Proc. Soc. Exp. Biol. and Med., 1945, **60**, 196.

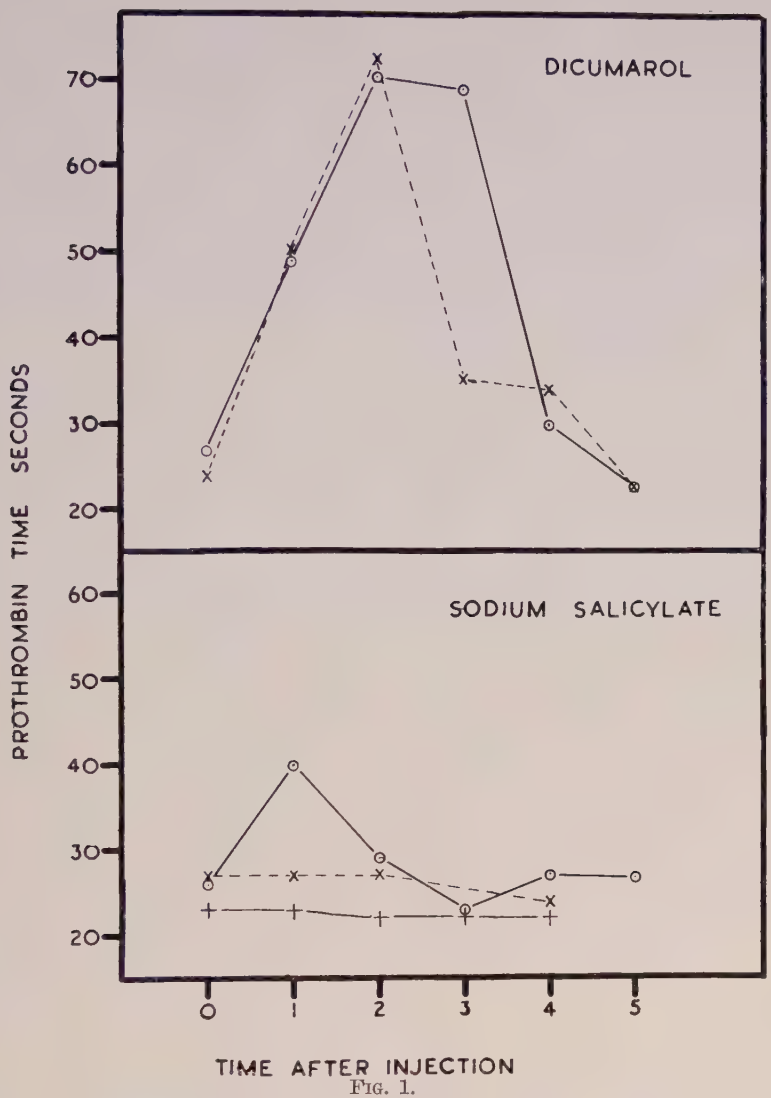

FIG. 1.

Effect of sodium salicylate and dicumarol on prothrombin time in rabbits. Rabbit 16. 6 mg of dicumarol, 0.8 g of sodium salicylate. —○—○— Oral; ——×——×—— Intravenous; —+—+— 1 g of sodium salicylate + 2 g of sodium sulfasuccidine orally.

control). The prothrombin response of each rabbit to 6 mg of dicumarol and 0.5 g/kg of sodium salicylate was determined both on oral and intravenous administration. The response was also determined after the oral administration of 2 g of sodium sulfasuccidine and of 2 g of sodium sulfasuccidine plus 0.5 g/kg of sodium salicylate. The sodium sulfasuccidine was given in 4 divided doses at 9 a.m. and 4 p.m. on 2 successive days, the sodium salicylate being given at 2 p.m. of the second day. After the experimental period of 5 to 6 days, a period of 4 days to several weeks was allowed before testing the next dose. The responsiveness of the animals was checked at intervals in the course of the experiment with the standard dose of dicumarol intravenously. A similar series was attempted using acetylsalicylic acid but it was found that the dose required orally to affect the prothrombin time was above the lethal dose when given intravenously.

TABLE I
Peak Prothrombin Times (12.5 % plasma) of Rabbits after Administration of Dicumarol and Sodium Salicylate.
secs.

Rabbit	Salicylate			Dicumarol	
	Oral	Intrav.	+ sulfa-succidine.	Oral	Intrav.
6	31.0 (26.5)	26.8 (26.7)		69.2 (24.8)	71.4 (26.3)
11	26.8 (25.0)	25.2 (26.3)	22.4 (21.8)	45.0 (24.8)	65.0 (26.2)
12	27.0 (24.8)	26.2 (26.3)	22.0 (21.8)	70.2 (25.9)	72.3 (22.6)
13	31.0 (27.2)	26.5 (26.4)		32.0 (25.0)	47.5 (26.3)
16	40.0 (27.2)	27.8 (27.3)	23.0 (21.8)	70.2 (25.9)	72.3 (22.6)

( ) = control prothrombin time on normal rabbit.

Results and Discussion. The prothrombin time response to the oral and intravenous administration of dicumarol and of sodium salicylate and to the oral administration of sodium salicylate + sodium sulfasuccidine are shown in Fig. 1. The peak prothrombin times obtained in 5 rabbits with these substances are reported in Table I. A definite increase in prothrombin time was observed after the oral administration of sodium salicylate. However, with the same dose administered to the same rabbit intravenously, no change in the prothrombin time was observed, although as previously reported by many investigators, the prothrombin response to dicumarol given intravenously to the same animal was of the same extent as after oral administration. Since Vitamin K was not withheld from the diet, the oral administration of sodium sulfasuccidine did not affect the prothrombin time. However, as shown in Table I, it did prevent the increase in prothrombin time after the administration of sodium salicylate.

The most direct explanation of the difference in the results on oral and intravenous administration of sodium salicylate is conversion of salicylate to dicumarol or a similarly acting compound in the intestinal tract. The action of sodium sulfasuccidine in abolishing the prothrombopenic action of salicylate suggests that this conversion is due to bacterial action. Attempts have been made to demonstrate such conversion with intestinal contents *in vitro,* by isolation of the products. This was unsuccessful. It may have been due to adverse conditions for bacterial synthesis or due to the small amounts of material involved. The prothrombopenic action of 1 g of sodium salicylate in the rabbits was equivalent to that of approximately 2 mg of dicumarol, so assuming formation of this compound, less than one per cent of the salicylate underwent conversion.

These results are somewhat at variance with those of Link[1] who reported that intravenous administration of sodium salicylate to rats did cause an increase in prothrombin time. This difference in results may be due to the use of a different animal species and also may be related to the vitamin K deficiency produced in the rats by Link. Further, Link does not give direct comparisons of oral and intravenous administration and his results are the average response of 6 rats. As originally shown by Link[1] on rabbits, marked differences occur in the response of individual animals to dicumarol (and also salicylate, Table I), and this is accentuated on a vitamin K-deficient diet. The results of Clark and Spitalny, of Stefanini and Petrillo, and the results reported here suggest that there are 3 possible mechanisms operative in the increased prothrombin time after the administration of salicylates, namely, a general action related to their analgesic-antipyretic properties, a direct effect on the clotting system of the blood, and lastly, conversion in the intestinal tract to dicumarol or a similarly acting compound.

Summary. The effect on the prothrombin

time of 12.5% plasma, of oral and intravenous administration of sodium salicylate, and of dicumarol has been compared in rabbits on a normal diet.

The oral and intravenous administration of dicumarol gave the same prolongation of the prothrombin time. However, while the oral administration of 0.5 g/kg of sodium salicylate definitely prolonged the prothrombin time, the intravenous administration of the same dose in the same animal did not result in any change in the prothrombin time. After the oral administration of sodium sulfasuccidine, the oral administration of sodium salicylate did not affect the prothrombin time.

It is suggested as an explanation of these results that salicylate may be converted to dicumarol or a substance with similar prothrombinopenic properties by bacterial action in the intestinal tract.

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Quantitative Aspects of the Inhibition of Anaphylactic Shock in Guinea Pigs.

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While the modifying action of the proprietary compounds β-dimethylaminoethyl benzhydryl ether hydrochloride (benadryl) and N′-pyridil-N′-benzyl-N-dimethylethylenediamine (Pyribenzamine) on histamine and anaphylactic shock has been vigorously investigated,[1] some conflicting data have appeared in the literature with regard to the action of these drugs on true anaphylactic shock in guinea pigs. Mayer, *et al.*,[2] demonstrated that 5 guinea pigs injected with horse serum 21 days before, were protected against an intracardial shock dose of 0.5 cc of horse serum when they were previously treated subcutaneously with 1.0 mg/kg of pyribenzamine. Campbell, *et al.*,[3] reported that although benadryl offered effective protection against histamine shock in the rabbit, it was ineffective in controlling anaphylaxis in actively sensitized (hen egg white) rabbits and guinea pigs. These latter workers used shock doses of 0.75 cc of the antigen for guinea pigs, given intraperitoneally. They conclude that their results seem at direct variance with those of Loew and Kaiser[4] and of Friedländer, *et al.*,[5] since these groups had found, similarly to Mayer's group,[2] that benadryl offers marked protection against anaphylaxis in the guinea pig.

Since in none of this previous work on benadryl and pyribenzamine cited was the attempt made to put protection against anaphylaxis in the actively sensitized animal on a quantitative basis, similar experiments were repeated with this view in mind.

Materials and Methods. The whites of 3 hen eggs were separated from the yolks, strained through cheesecloth into a beaker and then stored in a rubber stoppered vaccine bottle in the refrigerator. This material (Pro-

* The Rackham Arthritis Research Unit is supported by the Horace H. Rackham School of Graduate Studies of the University of Michigan.

1 Feinberg, S. M., *J. A. M. A.*, 1946, **132**, 702.

2 Mayer, R. L., Huttrer, C. P., and Scholz, C. R., *Science*, 1945, **102**, 93.

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tein = 11.53 g/%; NPN = 21.66 mg/%†) served as the source of sensitizing and shocking antigen throughout these experiments. The guinea pigs used weighed between 300 and 400 g. They received sensitizing injections of 0.1 and 0.2 ml of the egg white intraperitoneally and were kept in large common cages until ready for use. Intracardial injections were made using tuberculin syringes with No. 20 or 21, one or one and one-half inch needles. No anesthetic was employed. After proving the presence of the hypodermic needle in the heart by withdrawing 0.2 to 0.4 cc of blood, the egg white and blood mixture was reinjected slowly, within 30 seconds.

Dilutions of the egg white were made after centrifuging 3 to 4 cc of the refrigerated stock supply. The dilutions were made in saline with 1.0 ml serological pipettes graduated in hundredths and were in the range of the probable lethal dose (L. $D._{100}$) as determined by earlier experience.

The pyribenzamine and benadryl used were the commercial preparations put up for oral use and were incompletely soluble in 0.9% sodium chloride solution. These preparations were kept in test tubes and shaken to insure homogeneous suspension before placing in a syringe for intraperitoneal injection.

TABLE I

Determination of LD_{100} and Maximum Non-Lethal Dose of Egg White Required to Shock Actively Sensitized Guinea Pigs (19–21 days; 300–400 g) by Intracardial Injection.

No. of Animals	Antigen Dilution ml	Result*
1	.1 of 1:16	0
8	.1 1:8	1, 1, 1, 1, 1. 1, 1, 2
3	.2 1:8	3, 3, 3
6	.1 1:4	3. 3, 3, 3, 3, 3

* Results of intracardial injection were numbered as follows:

0, no symptoms noted.

1, mild symptoms. Face washing, coughing or sneezing, animal survives.

2, more severe symptoms, strong inspiratory movements, incontinence, animal survives.

3, severe symptoms and death within 3–8 minutes. Massive emphysema of lungs at autopsy.

4, severe symptoms, prostration followed by death after a delayed period of 15 to 60 minutes or more. Lungs not markedly emphysemic, but show some congestion. Congestion of kidneys, adrenals and mesenteric blood vessels. Beet-red appearance of stomach and intestinal outer wall. Lining of peritoneum dark pink to red.

† Nitrogen determinations (Kjeldahl) were made after concluding the experiments. The egg white was inadvertently frozen before the nitrogen determinations were made.

Results. Table I illustrates a typical titration to determine the L. $D._{100}$ of the egg white antigen used in these experiments. Among this group of animals sensitized 19, 20, or 21 days previously, none survived the intracardial injection of 0.1 ml of a 1:4 dilution or its concentration equivalent and none died of the effects of 0.1 ml of a 1:8 or higher dilution. The conclusion reached was that 0.1 ml of a 1:4 dilution represented an L. $D._{100}$ dose.

Table II shows the results obtained when sensitized guinea pigs, protected by pyribenzamine were injected with the same dilutions of egg white on the same day that the control animals were injected. These animals were injected intraperitoneally with the pyribenzamine 10 to 30 minutes preceding the shocking dose of egg white. They received either 5 or 10 mg of the drug with no attempt being made to vary the drug on a weight basis. Among 8 guinea pigs receiving 8 L. $D._{100}$ doses of egg white, 2 died after a delayed period and necropsy showed what appeared to be the systemic result of anaphylaxis in the guinea pig. The other 6 animals exhibited mild to severe symptoms, but lived in each case. Among 4 animals receiving less than 8 L. $D._{100}$ doses (*i. e.*, 1, 4, and 6) none exhibited symptoms of any type. Among 3 animals receiving more than 8 L. $D._{100}$ doses (*i. e.*, 20, 12, 10) all died within 7 minutes, in every way similarly to the control animals.

Table III illustrates the results obtained with sensitized guinea pigs protected against shock by benadryl which was intraperitoneally injected 15 to 30 minutes preceding the dose of egg white. Again, 5 or 10 mg of the drug was given with no effort being made to vary dosage according to weight of the animal. Among 4 animals receiving 8 L. $D._{100}$ doses none died although all exhibited mild to severe symptoms. One animal injected with 10 L. $D._{100}$ doses 20 minutes after receiving 10 mg of benadryl had violent symptoms but survived the shock and lived. Five others

TABLE II
Effect of Pyribenzamine on Amount of Egg White Required to Shock Actively Sensitized Guinea Pigs (300–400 g) by Intracardial Injection.

Animal No.	Days after Sensitization	Antigen Dilution ml	No. of Doses LD_{100}	Premedication: Intraperitoneal Dose mg	Premedication: Minutes Shocking Before	Result*
1	19	.1 of 1:4	1	5	10	0
2	19	.4 1:4	4	5	15	0
3	19	.4 1:4	4	5	20	0
4	19	.8 1:4	8	5	15	2
5	19	.8 1:4	8	5	15	4
6	19	.4 1:2	8	5	15	1
7	19	.4 1:2	8	10	15	4
8	19	.8 1:4	8	10	20	1
9	19	.4 1:2	8	10	20	1
10	19	.5 1:2	10	5	30	3
11	19	.6 1:2	12	10	15	3
12	19	1.0 1:2	20	5	20	3
13	20	.6 1:4	6	10	30	0
14	20	.8 1:4	8	10	15	1
15	20	.4 1:2	8	10	20	1

*Code same as that used in Table I.

TABLE III
Effect of Benadryl on Amount of Egg White Required to Shock Actively Sensitized Guinea Pigs (300–400 g) by Intracardial Injection.

Animal No.	Days after Sensitization	Antigen Dilution ml	No. of LD_{100} Doses	Premedication: Intraperitoneal Dose mg	Premedication: Minutes Before Shocking	Result*
1	20	.1 of 1:4	1	5	20	0
2	20	.4 1:4	4	5	25	0
3	20	.5 1:2	10	5	35	3
4	21	.4 1:4	4	10	20	0
5	21	.6 1:4	6	10	20	0
6	21	.4 1:2	8	5	15	2
7	21	.4 1:2	8	10	25	1
8	21	.4 1:2	8	10	30	1
9	21	.4 1:2	8	10	15	1
10	21	.5 1:2	10	10	20	2
11	21	.5 1:2	10	10	25	3
12	21	.6 1:2	12	5	25	3
13	21	.6 1:2	12	5	25	3
14	21	.6 1:2	12	10	25	3

*Code same as that used in Table I.

which received more than 8 L.D.$_{100}$ doses (*i. e.*, 12 or 10) all died within 7 minutes, exactly as had the control animals. Four animals receiving less than 8 L.D.$_{100}$ doses (1, 4, 6) exhibited no symptoms of shock.

Summary and Conclusions. The L. D.$_{100}$ dose of a solution of hen egg white by intracardial injection was determined for a group of actively sensitized guinea pigs. It was found that 5 to 10 mg of pyribenzamine or benadryl injected intraperitoneally between 10 and 30 minutes before giving the shocking dose protected the animals against approximately 8 L. D.$_{100}$ doses of the antigen while injection of 10 or more L. D.$_{100}$ doses resulted in rapid death with symptoms indistinguishable from the control animals. If 6 or less L. D.$_{100}$ doses were given, no symptoms were noted in the protected group.

These results seem to indicate that the so-called anti-histaminic drugs do exert a protective influence, quantitative in nature, against anaphylaxis in the actively sensitized guinea pig. This fact might well be expected since a similar quantitative protection is obtained with these drugs against histamine

shock.[1,2,3] The modifying action of these drugs again seems in accord with the theory that histamine plays a major role in anaphylaxis. It is of interest that approximately the same degree of protection was offered, in these experiments, by both the compounds used.

16028

Immunization of Mice Against Viruses of the Psittacosis Group with Ultraviolet-Inactivated Vaccines.*

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Various workers have demonstrated[1-4] that formalinized suspensions of psittacosis virus possess antigenicity as judged by their capacity to elicit active immunity in mice and avian species. No report has appeared, to our knowledge, of the effect of ultraviolet rays on this or other viruses of the psittacosis group. Levinson, Milzer, and co-workers, employing a new method of inactivating turbid suspensions of viruses and bacteria by ultraviolet rays, have found that it is possible to prepare experimental vaccines[5-7] with dysentery bacilli, and rabies, St. Louis encephalitis, and poliomyelitis viruses with a minimal loss of antigenicity. Briefly, this technic consists of exposing a continuously flowing thin film of suspension to the Oppenheimer-Levinson type lamp which is a powerful source of total and extreme (below 2000 Angstroms) ultraviolet energy. This method of inactivation was employed in the present study with the viruses of psittacosis (6BC), human pneumonitis (SF), and ornithosis (207).

Previous experiments[8] have indicated that these viruses can be completely inactivated by this method and still retain antigenicity as determined by the production of neutralizing antibody in chickens repeatedly inoculated with these preparations. It was our purpose here to make a preliminary investigation of the possibility of using such preparations for the induction of resistance against challenge injections of active virus.

Materials and Methods. The strains of virus employed have been used in previous work in this laboratory and their sources have been recorded elsewhere.[9] Earlier studies[10-12] have shown that large amounts of virus in relatively clear suspensions can be obtained by harvesting the allantoic fluid from embry-

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onated eggs inoculated into the allantoic cavity with certain members of this group. Consequently, this method of cultivation was utilized in the preparation of agents used as vaccines in the present work. Infected allantoic fluids were pooled, filtered through sterile gauze pads and stored in the dry ice-box until needed. Immediately prior to irradiation, the frozen materials were thawed at 37°C and filtered again to remove the small amount of insoluble flocculent precipitate frequently present.

In order to determine the least amount of irradiation necessary to inactivate the agents completely, an allantoic fluid preparation of each virus was divided into several portions which were irradiated for different periods of time. The exposure periods employed varied by 0.05-0.10 seconds and ranged from 0.05-0.30 seconds. It was found that periods of 0.10, 0.15, and 0.20 seconds were required to inactivate our preparations of psittacosis, human pneumonitis, and ornithosis viruses, respectively. A larger quantity of each virus was then titrated by intracerebral inoculation of mice, and inactivated by exposure to ultraviolet irradiation for the minimal time necessary as determined by the preliminary trials. Complete inactivation was confirmed in each instance by the tests described below. The LD_{50} titers,[13] by intracerebral test, before irradiation of the preparations finally used for immunization, were 0.03 cc x $10^{-6.3}$, $10^{-6.5}$, and $10^{-5.8}$, respectively, for the viruses of psittacosis, human pneumonitis, and ornithosis. Inactivated virus was stored in the dry ice-box, without the addition of a preservative, until used for the immunization of mice.

Rigorous tests for complete inactivation of irradiated virus were employed. They consisted of 3 serial intracerebral passages in mice and 3 serial allantoic passages in embryonated eggs. If these passages were all negative for active virus, 3 serial passages were performed by the intranasal route in mice and by the yolk sac route in embryonated eggs. In every instance, when active virus was present, as in the preliminary trials, it was demonstrated in either the first or second serial intracerebral or allantoic passage. Evidence suggested that the intracerebral route in mice and allantoic route in eggs were equally sensitive in the detection of active material.

Mice were vaccinated intraperitoneally with 2 and 3 0.5 cc injections of undiluted irradiated virus given at 5- or 7-day intervals. The challenge dose of homologous virus consisted of suspensions of infected mouse brain or pooled liver and spleen tissue, and was given either intracerebrally or intraperitoneally to groups of mice together with controls, 3 weeks after the terminal injection of vaccine. The LD_{50} titers of these suspensions were determined before use by intracerebral or intraperitoneal inoculation of mice, depending upon the tissue source.

The emulsion of mouse brain passage virus, first used for intraperitoneal challenge of mice (those which received 2 injections of psittacosis vaccine, Table I) was not sufficiently virulent by this route to give regular, clear-cut results. An attempt was made to overcome this circumstance by performing several rapid serial intraperitoneal passages with emulsions of pooled virus-infected liver and spleen tissue. A definite enhancement in virulence was obtained after 8 passages as indicated by a decrease from 7 to 2 days in the average survival time of animals inoculated with 10% emulsions. An emulsion of pooled infected liver and spleen tissue was then prepared, titrated, and used as the challenge inoculum in the experiment where 3 injections of psittacosis vaccine were employed.

Results. The results shown in Tables I and II indicate that a definite degree of protection was demonstrable in most experiments. Little or no resistance was seen in mice challenged intracerebrally with psittacosis virus (Table I), but definite immunity was demonstrated in psittacosis-vaccinated mice when the challenge dose was given intraperitoneally. Evidently this resistance was not due in part to non-specific immunity since no protection was seen in mice which had received three intraperitoneal injections of normal allantoic fluid three weeks before the challenge dose of

[13] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

TABLE I
Resistance of Mice Immunized with Ultraviolet-Inactivated Psittacosis Virus.

Injections of vaccine	Challenge Injection			Result†	
	Volume	LD_{50} Doses	Route	Vaccinated mice	Non-vaccinated controls
2	0.03 cc	4,000	Intracerebral	12/12	6/6
"	"	400	"	12/12	6/6
"	"	40	"	12/12	6/6
"	"	4	"	6/12	6/6
"	0.03 cc	40,000	Intraperitoneal	2/3	6/6
"	"	4,000	"	0/10	4/6
"	"	400	"	0/10	4/6
"	"	40	"	0/10	4/6
3	0.25 cc	48*	"	15/38	27/27 26/26**

†Numerator indicates number of deaths; denominator indicates number of mice inoculated.
*LD_{50} titer determined here by intraperitoneal inoculation; all others determined by intracerebral.
**This set of controls received normal allantoic fluid at times of immunization.

TABLE II
Resistance of Mice Immunized with Ultraviolet-Inactivated Human Pneumonitis and Ornithosis Viruses.

Virus	LD_{50} Doses of Challenge Injection (0.03 cc, intracerebral)	2 inj. of vaccine		3 inj. of vaccine	
		Vaccinated	Control	Vaccinated	Control
Human Pneumonitis	180	5/8	8/8	4/10	8/8
	18	0/7	8/8	0/10	8/8
Ornithosis	900			8/10	6/6
	90	3/9	6/6	1/10	6/6

virus was given. Resistance to intracerebral challenge was definitely demonstrated in mice which had received human pneumonitis and ornithosis vaccines (Table II).

Conclusions. These studies demonstrate that it is possible to inactivate completely certain viruses of the psittacosis group in less than one second by exposure to appropriate intensities of ultraviolet irradiation. Such preparations retain antigenicity as indicated by their ability to immunize mice against challenge doses of active virus.

16029 P

Techniques for Rickettsial and Virus Cultivation.

JOZEF KUBICZ. (Introduced by R. W. G. Wyckoff.)

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The following note describes modifications made in the Weigl method for cultivating rickettsiae and other small agents of disease within susceptible lice. Made primarily to facilitate isolations from diseased persons, they involve feeding larvae reared under sterile conditions on potentially infected blood.

The technique is essentially as follows. From 200-400 adult lice (*Pediculus vestimenti*) are washed for 2 minutes in 60% alcohol and subsequently with sterile physiological saline. They are then enclosed in a cage (6 x 3 x 1 cm) covered on one side with a fine net and containing within it the piece of

cloth recommended by Weigl. The caged lice are fed twice daily on a healthy nourisher whose skin is first sterilized with 70% alcohol. Between feedings the cages are kept in sterile paper boxes at 34°C. Under these circumstances, masses of eggs are laid on the cloth. These eggs are removed and washed first with 30% alcohol or other suitable weak disinfectant and then with sterile saline, bacterial sterility of the washed eggs being checked by culturing the last drops of the saline wash on nutrient agar. The sterile eggs are dried on sterile paper and incubated at 34°C in sterile net-covered cages, 200-400 eggs per cage. The eggs hatch after 4 to 6 days and the larvae are then ready for use.

When used to isolate rickettsiae or other agents of disease from a patient, such a cage of larvae is fed sterilely and twice daily over a period of four to twelve days, depending on the disease being investigated. To do this, the net side of the cage is attached to the sterilized skin on the medial side of the forearm or shank. Sterilization is affected with ether followed by 70% alcohol, care being taken to be sure that the alcohol has completely evaporated before feeding begins. Bacterial sterility is routinely checked by seeding larval excrement to agar plates.

At the conclusion of the 4- to 12-day feeding period, the cage is opened and note made of the living and dead larvae, especial attention being given those that are red or red-black in color. Infectivity is sought in the accumulated excrements and in the sterilely resected intestines of the living and dead reddish larvae. Microscopic examination is carried out with the aid of contrast methods of Serkowski[1] (Nigrosin, 4% in H_2O) or by the Eisenberg[2] method.

In this way, we have isolated and observed the infectious bodies responsible for a number of virus and rickettsiae-like diseases. These bodies are, for the most part, round disks having about the size of typhus rickettsiae. Several of the diseases they produce are rheumatic in character (*Polyarthritis rheumatica acuta, Chorea minor*), though one gives a toxic diarrhea in infants. To identify what is seen in stained preparations with the diseases in question, we have made use of both agglutination and of the Bordet-Gengou reaction. Some isolations have produced symptoms in rabbits and sensitized guinea pigs. Inactivated suspensions have given as intracutaneous test prominent urticaria-like reactions in persons sensitized through chronic disease and have thus proved a useful means of diagnosis. In sensitive persons, this reaction also supplies an excellent test for the presence of traces of the agents in louse intestines. At all times, corresponding emulsions of healthy louse intestines have been used as controls.

An essentially similar procedure for isolating infectious material has used bed bug (*Climicis lectularii*) instead of louse larvae. It often permits the isolation of agents that would be missed using louse larvae. In this case, small pieces of wood replace cloth as receivers of laid eggs and feeding is once a day for an hour. Each cage is set up for 60-80 eggs. Incubation takes place at a lower temperature (28-30°C).

This work was begun in the Typhus Institute of Professor R. Weigl and in the Children's Clinic of Professor Fr. Groer in Lwow. It has been continued in the Dermatologic Clinic of Professor Lenartowicz in Wroclaw.

1 Sterling-Okuniewski, *Technika badan bakteriologicznych* (Warzawa, 1922), p. 72.

2 Eisenberg, Ph., *Zentr. Bakt. I akt Originale Bd.*, **71**, 421.

16030

Blood, Plasma, and "Drawn Blood" Volumes in the Rat.

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The following simple method for determination of circulating blood and plasma volumes has been used in the normal albino rat. It can be applied usefully to control and experimental rats just before they are killed.

The rat was anesthetized with ether in a Mason jar. As soon as the animal ceased to move, the inferior vena cava was exposed rapidly by cutting the abdominal cavity widely open with scissors. Light anesthesia was continued by applying a small beaker containing ether-soaked cotton over the nose. A hemoglobin solution of known volume and concentration was injected slowly into the vena cava from a tuberculin syringe.† The volume was varied from 0.20 to 0.50 cc, depending upon the size of the rat, and the concentration varied from 7% to 8%. The needle was kept in place to prevent leakage for two minutes. It was then withdrawn and the animal was exsanguinated by severing the abdominal aorta. The first ml of blood was collected in a tube containing 2 mg of potassium oxalate, for a hematocrit determination by the Wintrobe method. The remaining blood was collected carefully, until all bleeding stopped, in an oiled centrifuge tube and allowed to clot. Under these conditions we had previously found that hemolysis did not occur.

The total "drawn blood' was recorded. The clotted blood was centrifuged and the serum separated for measurement of its oxyhemoglobin concentration by the method of Evelyn and Malloy.[1] From these data plasma and blood volumes were calculated and corrected by subtracting the volume of hemoglobin solution injected.

The procedure outlined was selected, in spite of known objections, because it happened to fit our experimental needs, and required simple analytical techniques with which we were fully familiar. Essentially, it is a dye method in which blood is drawn for measurements of concentration and hematocrit after a suitable mixing time. Preliminary experiments showed that a 2-minute mixing time gave the same results as longer mixing times. Since the interval was short and the dye was one that is essentially nondiffusible and relatively slowly metabolized, use of a mixing curve, such as that of Noble and Gregerson[2] seemed unnecessary and technically would be almost impossible to obtain.

It was also found that skill of the operator was of some importance in obtaining reproducible results. At first determinations will tend to be low, presumably due to shock resulting from slow and inexpert handling of the animals. However, with a reasonable amount of practice, relatively constant and repeatable results are obtained.

Results. Blood volumes (BV) and plasma volumes (PV) were determined in 125 rats (47 female, 78 male) ranging from 48 to 308 g. In 111 the "drawn blood' volume was recorded. As there did not appear to be any significant difference between the sexes, the results were pooled. Plots of PV and BV against body weight in grams (BW) and body surface in square centimeters (BS) do not produce a straight line, but when log BV and log PV are plotted against log BW (Fig. 1) the points fit a straight line reasonably well. From the data obtained, regression lines were

* The author wishes to make grateful acknowledgment of the assistance of Helen J. Ureen. This work was aided by a grant from the American Medical Association.

† Hemoglobin solution provided through the courtesy of Sharpe & Dohme, Inc., Philadelphia, Pa.

1 Evelyn, K. A., and Malloy, H. T., *J. Biol. Chem.*, 1938, **126**, 655.

2 Noble, R. P., and Gregerson, M. I., *J. Clin. Invest.*, 1946, **25**, 158.

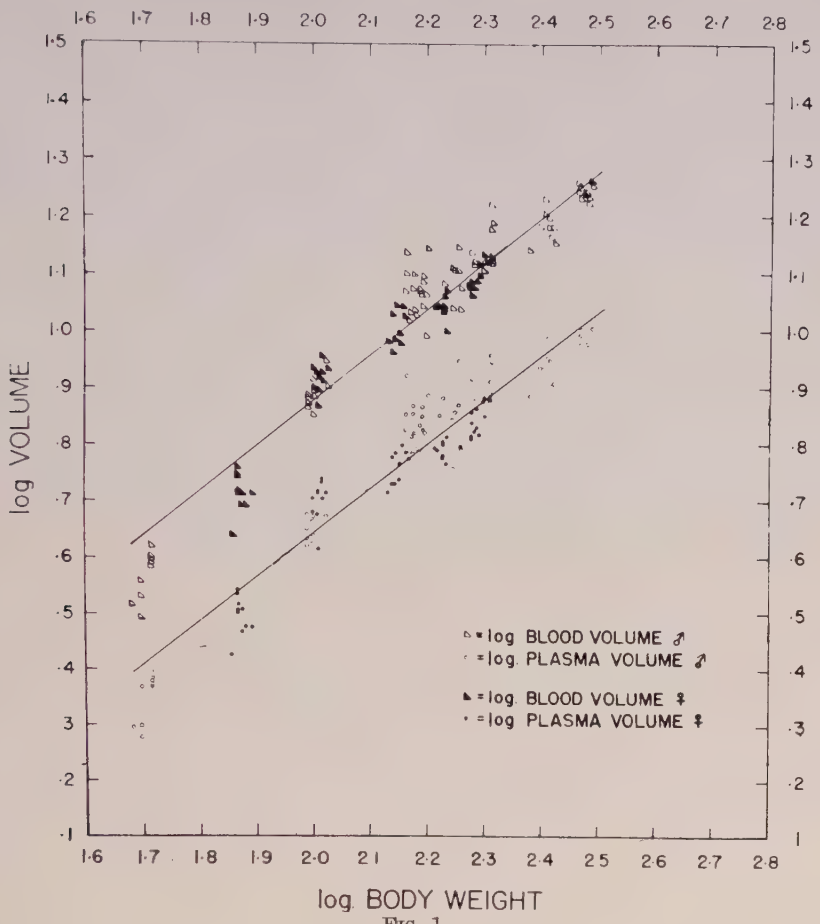

FIG. 1.

calculated, and the following formulae were established as applicable to the observed weight range:

$$BV = 0.195\ BW^{0.794}$$

$$PV = 0.122\ BW^{0.778}$$

Nearly all of the observations fell within a reasonably small zone on either side of the line, the values falling below the line at weights below 100 g. Although the relation of blood and plasma volume to a power of body weight was not perfect, it was a better fit than any simple alternative, and is presented for purposes of comparison with other investigators' results.

The general relationship of organ size to a power of body size has been established by Huxley,[3] who used body size from which the size of the organ has been subtracted. The use of total body weight is of considerably greater convenience and in the present instance, where the weight of the blood is a relatively small proportion of the total body weight, the difference introduced by such use was found to be insignificant. Failure of the regression line to fit more perfectly may be due to increased deposition of fat, a relatively avascular tissue, as the rats approach maturity and senescence.

Comparison of our results with those published previously is complicated by the various methods of presenting data. Table I summarizes in chronological order the results of several different observers and, for purposes of comparison, gives the blood and plasma volumes calculated by us for a rat of 150 g. In the references cited, body surface

[3] Huxley, J. S., *Problems of Relative Growth*, New York, The Dial Press, 1932.

TABLE I.

Author	Formulæ BV	Formulæ PV	150 g rat BV cc	150 g rat PV cc
Chisolm[5]	.099 $BW^{0.9}$		9.0	
Went and Drinker[6]	.074 BW		11.1	
Cutting and Cutter[7]		.014 BS		4.5
Griffith and Campbell[8]	.043 BW		6.5	
Beckwith and Chanutin[9]	.054 BS	.027 BS	17.2	8.7
Metcoff, Favour and Stare[10]	.047 BS	.028 BS	15.2	8.7
Lippman	.195 $BW^{0.794}$	.122 $BW^{0.778}$	10.4	6.0

BV is blood volume in cc, PV is plasma volume in cc, BW is body weight in grams, BS is body surface in sq.cm.

was assumed to have been calculated by the formula of Carman and Mitchell[4] where not otherwise stated.

The "drawn blood" volumes obtained by measurement have been plotted in Fig. 2 against the blood volumes determined by the hemoglobin method described. The curve obtained indicates that the "drawn blood" volume is related to actual blood volume, being somewhat below 50% of the actual blood

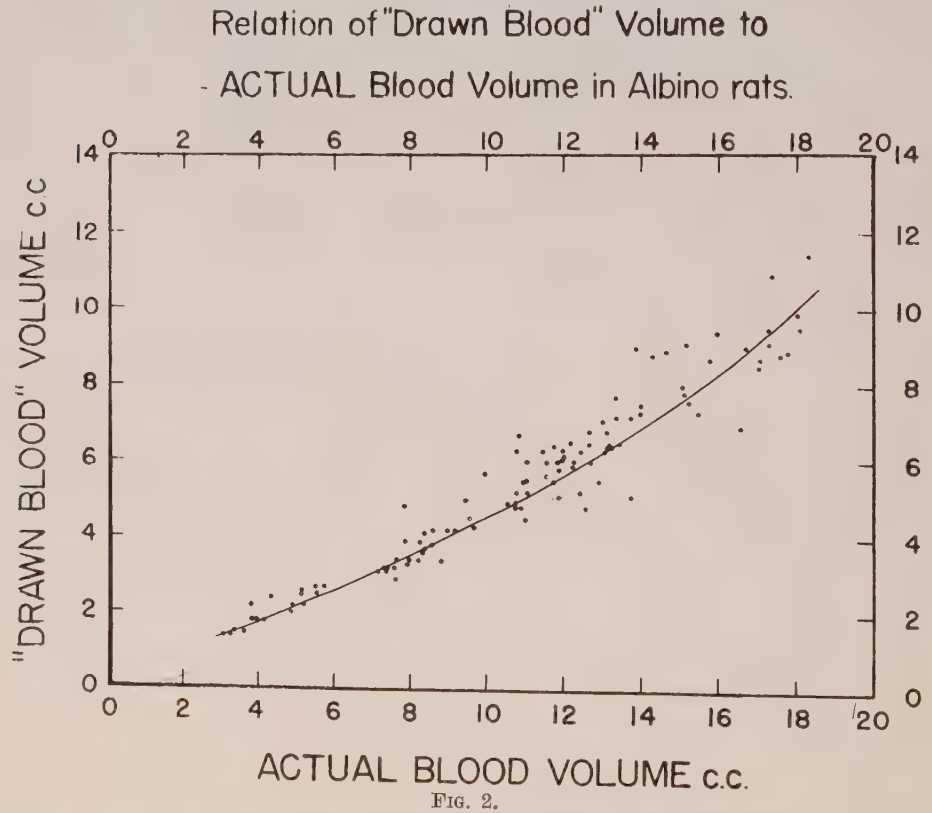

FIG. 2.

4 Carman, G. G., and Mitchell, H. H., *Am. J. Physiol.*, 1926, **76**, 380.

5 Chisolm, R. A., *Quart. J. Exp. Physiol.*, 1911, **4**, 207.

6 Went, S., and Drinker, C. K., *Am. J. Physiol.*, 1929, **88**, 468.

7 Cutting, W. C., and Cutter, R. D., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1053.

8 Griffith, J. P., Jr., and Campbell, R., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 38.

9 Beckwith, J. R., and Chanutin, A., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 66.

10 Metcoff, J., Favour, C. B., and Stare, F. J., *J. Clin. Invest.*, 1945, **24**, 82.

volume at weights below 100 g and somewhat above 50% at high weights. This confirms the impression acquired incidentally in this laboratory, in the process of accumulating data in numerous other rat experiments, that an indication of the direction of change in actual blood volume can be obtained by measuring the "drawn blood" volume.

Summary. 1. Blood and plasma volumes have been determined in the normal albino rat, and have been found to be approximately related to a power of body weight, the deviation being ascribed to deposition of avascular fat as the rat matures. There appeared to be no significant difference in this regard between the sexes.

2. An estimation of blood volume changes may be made from the "drawn blood" volumes.

16031

Effect of Chloroform on the Antitryptic Activity of Blood Plasma.

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It was shown in a previous paper[1] that treatment of plasma with 10% chloroform, decreased its hypertensinase activity instead of increasing it. This was a remarkable finding because: (a) hypertensin is a very sensitive reagent to test for proteolytic activity, and (b) the characteristic clotting and fibrinolytic properties of chloroform treated plasma have been attributed to an increased tryptic activity.

Previous experiments have also shown that hypertensin is an excellent substrate for the detection of antiproteolytic substances. Using hypertensin as a reagent, we have been able to show that plasma protects hypertensin against the destructive action of trypsin and to a lesser degree from that of chymotrypsin. On the contrary, the hypertensinase activity of crystalline carboxypeptidase and pure aminopeptidase (from yeast) remained unaffected (Croxatto and Croxatto[2]).

The degree of protection afforded by plasma, against the destruction of hypertensin by an appropriate dose of trypsin, may be used as a measure of its antitryptic activity. By using a technique based on this assumption, we have studied the effect of chloroform treatment on the antitryptic activity of plasma.

Experimental. Dog, ox, horse and human plasmas were treated with 10% chloroform, with the technique already described. Chloroform was separated by centrifuging and vacuum distillation, usually after 24 hours contact with the plasma. Aliquots of treated and untreated plasma were added to hypertensin (3 units) plus 0.1 ml of a solution of crystalline trypsin. A freshly prepared solution of one mg of trypsin* per milliliter of 0.9% sodium chloride was used. The amount of this solution used (0.1 ml) was enough to produce more than 80% destruction of hypertensin in 2 hours, with no plasma present.

The action of plasma on hypertensin, in absence of trypsin, was also tested. All experiments were carried out at pH 7.4, obtained by adding 0.2 ml of a solution of 0.02N sodium phosphate buffer, pH 7.4. The incubation period was of 2 hours at a temperature of 37°C.

The hypertensin activity present after incubation was determined by injecting the solutions into the femoral vein of a cat, under Dial anesthesia and recording with the

1 Sainz, N., and Croxatto, H., *Bol. Soc. Biol.*, Santiago, Chile, **1944, 2, 261.**

2 Croxatto, H., and Croxatto, R., *Bol. Soc. Biol.*, Santiago, Chile, in press.

* Plant's crystalline trypsin containing 85% of magnesium sulfate.

usual technique the changes in blood pressure in the carotid artery. All solutions were boiled and then centrifuged, using supernatant. This treatment eliminates completely the secondary effects of foreign proteins and trypsin on the blood pressure. The sensitivity of the animal was checked, from time to time, with a standard solution of hypertensin.

The amount of plasma, not treated with chloroform, necessary to afford a 50% protection (1.5 units of hypertensin) varied between 0.04 and 0.06 ml. The hypertensinase activity of such a volume of plasma was negligible.

Results. As shown in Fig. 1, the treatment of plasma with chloroform decreases greatly its antitryptic activity. Although chloroform treatment does not eliminate completely the antitryptic activity, the decrease is larger after 24 than after one hour contact. The antitryptic activity of 0.5 ml of 24 hours treated plasma is approximately equivalent to that of 0.05 ml of untreated plasma.

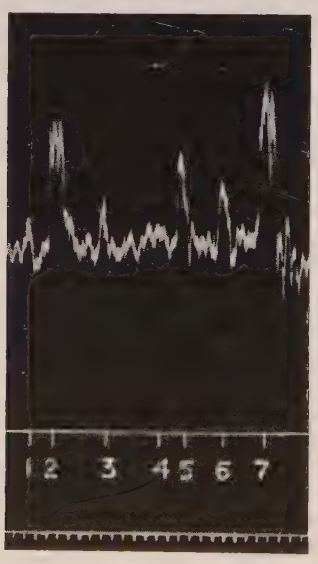

FIG. 1.

Discussion. Our results favor the hypothesis of an inactivation, by the chloroform treatment, of one or several substances acting as inhibitors of the pancreatic trypsin. Heparin cannot be considered as such an inhibitor because of not appreciably influencing the inactivation of hypertensin and pepsitensin by trypsin, whether plasma is present or not (Marsano, Croxatto, Croxatto[3]).

The clotting and proteolytic activities of chloroform treated plasma may be due to an inhibition of antitryptic substances. In fact, the addition of crystalline antitrypsin extracted from soy bean meal, counteracts the clotting and fibrinolytic action of chloroform treated plasma and certain snake venoms (H. Croxatto[4]).

The changes produced by the chloroform treatment are very complex and difficult to interpret. Several enzymatic activities of plasma are disturbed (Sainz and Croxatto[1]). and the interaction of hypertensinogen, renin, and hypertensinase is changed (Croxatto and Croxatto[2]). Furthermore, no explanation can be given of why hypertensin is destroyed more easily by non-treated plasma than by plasma treated with chloroform, while the proteolytic activity is increased by this treatment, as shown by the solubilization of the blood clot.

Summary. Treatment of human, dog, ox, and horse plasmas with 10% chloroform, decreases their antitryptic properties.† These properties were measured using hypertensin as substrate.

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3 Marsano, A., Croxatto, R., and Croxatto, H., *Bol. Soc. Biol.*, Santiago, Chile, 1945, **2**, 350.

4 Croxatto, H., *Rev. Soc. argent. Biol.*, 1946, **22**, 477.

† While this work was in course of publication, a paper by L. R. Christensen appeared in *J. Gen. Physiol.*, 1946, **30**, 149, where he states that chloroform treatment of serum produces an immediate inactivation of "protease inhibitor."

16032

The Serum Uric Acid Concentration in Essential Hypertension.*

JOSEPH R. STANTON AND EDWARD D. FREIS. (Introduced by Robert W. Wilkins.)

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The blood uric acid concentration in patients with arterial hypertension has not received careful attention since early investigators reported the uric acid level to be increased.[1-4] This reported increase was ascribed to metabolic factors and not to beginning renal insufficiency.[3] More recent work on methods for the determination of the serum concentration of uric acid indicated that values determined by earlier methods contained many sources of potential error.[5-8] Further, many of the subjects previously studied had varying degrees of cardiac and renal failure.

If there is a metabolic fault in essential hypertension that results in hyperuricemia it would be of importance as a lead to further investigation. However, it was considered necessary first to reevaluate the question using modern methods of analysis and paying particular heed to the presence of renal insufficiency.

Material and Method. Fifty consecutive hypertensive patients admitted to the Ward and Private services of the Massachusetts Memorial Hospitals for lumbo-dorsal splanchnicectomy were the subjects of this study (c. f. Table I). Chronic glomerulonephritis and other forms of primary renal disease were adequately ruled out by history and laboratory examinations.

TABLE I.

	No. of cases
Males	20
Females	30
Age group	
20-30	4
30-40	13
40-50	19
50-60	13
60-70	1
Systolic blood pressure over 180 mm Hg.	42
Diastolic blood pressure over 100 mm Hg.	48
Proteinuria	20
Nitrogen retention	2

Hypertension had been known to exist in these patients for from 6 months to 20 years. The highest blood pressure recorded on admission was 250/160, the lowest 160/96. Only one patient was in cardiac failure during the period of observation.

The determination of uric acid was carried out by the method of Folin,[7] adapted to the Klett Summerson photoelectric colorimeter† using a protein free filtrate of serum.[9] Blood samples from all patients in this series were taken in the post-absorptive state. Normal values obtained by this method ranged from 2-5 mg/100 cc of serum.

Results. Persistent hyperuricemia was demonstrated in only 2 cases or 4% of the series. Normal serum uric acid levels were found on the initial determination in 44 cases. Six cases exhibited a serum uric acid level above 5 mg % at the time of the initial determination. On repeat examination carried out 24 to 48 hours later, 4 of these 6 cases were found to have uric acid levels well within the normal

* This investigation was supported in part by the Squibb Institute for Medical Research, New Brunswick, N. J.

1 Kylin, E., *Acta med. Scandinav.*, 1923, **58**, 342.

2 Hitzenberger, K., Richter and Quinter, M., *Wien. Arch. f. inn. Med.*, 1921, **2**, 189.

3 Fishberg, A. M., *Arch. Int. Med.*, 1924, **34**, 503.

4 Williams, J. L., *Arch. Int. Med.*, 1921, **27**, 748.

5 Wu, H., *J. Biol. Chem.*, 1922, **51**, 21.

6 Folin, O., *J. Biol. Chem.*, 1934, **106**, 311.

7 Folin, O., *J. Biol. Chem.*, 1933, **101**, 111.

8 Dana, E. S., Bosnes, R. W., and Dill, L. V., *J. Clin. Invest.*, 1944, **23**, 776.

† General Directions for the Klett Summerson Colorimeter, Klett Mfg. Co., New York.

9 Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, **38**, 81.

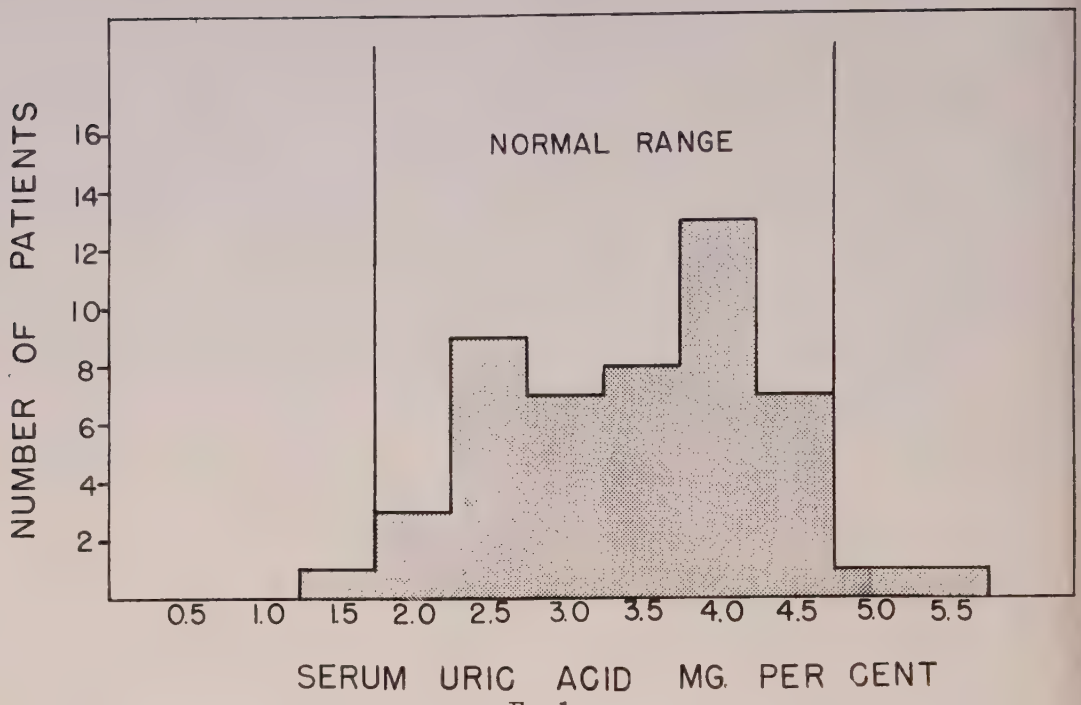

FIG. 1.
Serum uric acid concentration of 50 patients with essential hypertension.

range. The mean serum uric acid level for the entire series was 3.65 mg % with a standard deviation of 0.88 (Fig. 1).

Discussion. It is significant that the 2 cases with persistent hyperuricemia showed evidence of renal insufficiency as manifested by elevation of the blood non-protein-nitrogen. Proteinuria was present in both of these cases and also in 3 of the 4 cases showing transient hyperuricemia. However, hypertension and proteinuria were observed in 12 patients showing normal uric acid levels. Therefore, no consistent relationship was observed between proteinuria and elevated serum uric acid levels. The transient elevations of serum uric acid concentration observed in four cases were slight, none exceeding 5.5 mg %.

Summary and Conclusions. In a series of 50 cases essential hypertension, unless complicated by renal insufficiency, was associated with essentially normal serum uric acid values.

The authors are indebted to Miss Anne Cutler for valuable technical assistance.

16033 P

Kidney Damage in the Golden Hamster Following Chronic Administration of Diethylstilbestrol and Sesame Oil.*

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Estrogen induced hyperplasias have been reported repeatedly for various species of animals. The most conspicuous of such changes reported so far for the estrogenized hamster are in the hypophysis. In this gland the pars intermedia develops very extensive adenomas which migrate through the stalk well into the hypothalamus (Vasquez-Lopez,[1] Koneff, Simpson and Evans,[2] Matthews[3]). For the hamster the only kidney alteration mentioned in the literature is found in the paper by Vasquez-Lopez where Table I records a "large secondary deposit macroscopically visible in the left kidney" of one of 28 hamsters implanted with 10 mg pellets of diethylstilbestrol or of estradiol benzoate; this particular animal had been treated for 299 days with the natural estrogen.

While a tumor-producing action, in guinea pigs, has been denied for sesame oil by Lipschütz and Vargas[4] it has been affirmed in man by Conrad, Conrad and Weiss.[5] To date we have had no opportunity of examining the kidneys of sesame oil-treated control hamsters. We know of no reports in the literature, however, which attribute the formation of renal adenomas, in any species, to treatment with either estrogens, sesame oil, or any other similar oil.

In this laboratory we have examined kidneys from 14 hamsters (*Cricetus auratus*) in which group were 4 untreated males, one untreated female, 6 treated males and 3 treated females. On the average the treated animals received a total of 41 mg of diethylstilbestrol in 25 cc of sesame oil followed by a total of 61 mg in the form of microcrystals in 23 cc of saline solution. Injections were made subcutaneously, daily or on alternate days, over a period averaging 379 days. The animals averaged 429 days in age and 112 g in body weight at the time of sacrifice.

Kidney changes were observed in all of the 9 treated animals. Of these changes by far the most conspicuous were adenomas (Fig. 1C) found in each of the 6 males. In size these tumors ranged from small nodules a few cells in width to masses about one-third of the volume of a control kidney. In many of the adenomas were groups of cells showing "colloid degeneration", rare in the cells of intact tubules. Many of the adenomas were in the region of the pelvis, projecting more or less into the renal sinus. The cell arrangement in some of these suggests an origin from the transitional epithelium of the pelvis. Other adenomas occurred in various portions of the kidney cortex. The fact that the adenomas alone of all the kidney tissue contain much sudanophilic substance suggests that sesame oil (also sudanophilic) may be the tumor-producing agent. Experiments are under way to test the relative actions of oil and estrogen and to investigate the cause of the sex difference in kidney response. In one animal the renal tumor had metastasized through the peritoneal cavity to the mesenteries and the spleen.

In 2 males, fibromas were observed in the capsular connective tissue.

No extraglomerular arteriosclerosis was observed, but in the females amyloid infiltration

* The diethylstilbestrol used in this study was supplied through the courtesy of Dr. D. C. Hines of the Eli Lilly Company.

1 Vasquez-Lopez, E., *J. Path. and Bact.*, 1944, **56**, 1.

2 Koneff, A., Simpson, M. E., and Evans, H. M., *Anat. Rec.*, 1946, **94**, 169.

3 Matthews, V. S., 1947 unpublished data.

4 Lipschütz, A., and Vargas, L., *C. R. Soc. biol.*, 1939, **130**, 9; Lipschütz, A., Rodriguez, F., and Vargas, L., *C. R. Soc. biol.*, 1939, **130**, 939.

5 Conrad, A. H., Conrad, A. H., Jr., and Weiss, R. S., *J. A. M. A.*, 1943, **121**, 237.

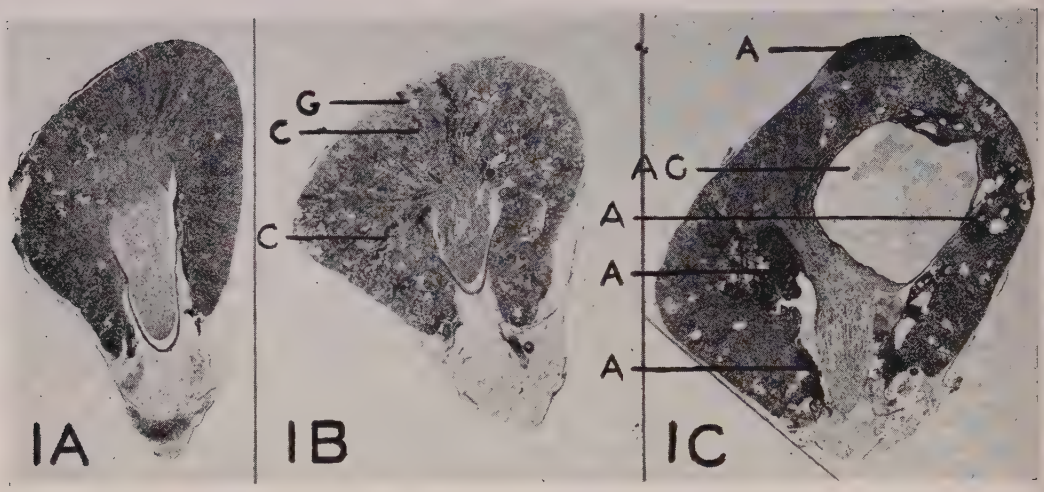

FIG. 1.

Fig. 1, A to C, (all ×5) are transverse sections through kidneys of golden hamsters. 1A represents a normal kidney from a 389-day-old ♀ weighing 136 g; untreated. 1B is from a 364-day-old ♀ weighing 85 g; received 29 mg of diethylstilbestrol in 17 cc of sesame oil over a period of 107 days, followed by 59 mg in the form of microcrystals suspended in 20 cc of saline solution over a period of 215 days. 1C is from a 456-day-old ♂ weighing 130 g; received 55 mg of diethylstilbestrol in 33 cc of sesame oil over a period of 205 days, followed by 58 mg of microcrystals in 19 cc of saline solution over a period of 215 days. A, adenoma; Ac, cyst in adenoma; C, casts in enlarged tubules; G, enlarged capsular space about glomerulus.

was considerable in many glomeruli and around many convoluted tubules. This was associated with large and abundant hyaline casts and tubular atrophy (Fig. 1B). In 2 of the 6 treated males also, amyloid was demonstrated. In one animal it was confined to traces in a few of the glomeruli; in the other animal, No. 49, (the one possessing fewest and smallest adenomas) it was present in somewhat greater amount and found around convoluted tubules as well as in glomeruli. A similar picture of glomerulo-nephritis has been described by Korenchevsky and Ross[6] in both male and female, normal and gonadectomized, albino rats treated with 0.018 to 0.2 mg of estradiol dipropionate, in sesame oil, weekly for from 21 days to 3¾ months. They make no mention of sesame oil controls or of sexual differences in kidney reaction.

A more detailed account, involving hamsters still being treated, will be published later.

Summary. The kidneys of golden hamsters treated for long periods with diethylstilbestrol and sesame oil tend to undergo marked changes of a destructive character. In the male these changes are primarily in the direction of potentially malignant tumor formation. In the female they are in the direction of glomerulo-nephritis

[6] Korenchevsky, V., and Ross, M. A., *Brit. Med. J.*, 1940, **1**, 645.

16034

Effect of Meningococcal Endotoxin on Histamine Content of Blood and Tissues of Rabbits.*

ERNEST KUN. (Introduced by C. Phillip Miller.)

From the Department of Pharmacology and the Department of Medicine, University of Chicago.

The following studies were undertaken as part of an investigation of the biochemical changes which occur in the animal body after the intravenous injection of meningococcal endotoxin. The pathological condition produced in this way is known as toxemia and since different bacterial toxins seem to elicit changes in metabolism which have certain characteristics in common,[1] the results of these studies may be of some general interest.

The important role of histamine in the pathology of infectious diseases, as well as in conditions closely related to them, such as allergy and anaphylaxis, is well recognized; and has been reviewed by Best[2] and by Dragstedt.[3,4] Feldberg and Keogh[5] and Feldberg and Kellaway[6] reported experiments which showed that an increase in histamine liberation from isolated organs occurred when the perfusion fluid contained staphylococcal endotoxin. Kellaway, Trethewie and Turner[7] showed that the toxin of *Clostridium welchii* increased the histamine output of isolated organs of the cat and the rabbit. These results suggest that bacterial toxins interfere in some way with histamine metabolism.

The present study is concerned with the changes in the histamine content of the blood and tissues of rabbits injected intravenously with meningococcal endotoxin.

Materials and Methods. The meningococcal endotoxin, as prepared by Boor and Miller, is a mixture of nucleoproteins, globulins, albumins, and a highly toxic glycolipid.[8,9,10] Endotoxin was prepared from type I meningococcus grown for 18 to 20 hours on casein-digest agar. The microorganisms were washed thrice in saline, resuspended in water, brought to pH 8.2 to 8.5 and kept at a temperature of about 27°C for 2 or 3 hours and in the refrigerator overnight. The suspension was then neutralized and sterilized by heating 2 or 3 times at 60° C for 25 minutes. Sterility was always proved by culture. This toxin mixture was heat stable,[11] and could be kept in the icebox for several months without much change in toxicity. Since the chemical nature of meningococcal endotoxin is not yet known, it seemed best to use the original protein-glycolipid suspension. The rabbits were injected intravenously with a dose sufficient to cause death within a few hours and this dose is recorded in ml per kg body weight. The solid content of the toxin was 1.14% as determined by Boor. These experimental conditions can be considered as acute meningococcal endotoxin poisoning.

* A preliminary report was presented before the Chicago meeting of the Federation of American Societies for Experimental Biology, May, 1947.

This investigation was supported in part by the John and Mary R. Markle Foundation and in part by the United States Navy, Office of Naval Research.

1 Holmes, E., *Physiol. Rev.*, 1939, **19**, 439.

2 Best, C. H., *Physiol. Rev.*, 1931, **11**, 371.

3 Dragstedt, C. A., *The Significance of Histamine in Anaphylaxis and Allergy*, 1943.

4 Dragstedt, C. A., *The Role of Histamine in Various Pathological Conditions and the Methods Controlling Its Effects*, 1945.

5 Feldberg, W., and Keogh, V., *J. Physiol.*, 1937, **90**, 280.

6 Feldberg, W., and Kellaway, C. H., *Australian J. Exp. Biol. Med. Sci.*, 1938, **16**, 219.

7 Kellaway, C. H., Trethewie, E. R., and Turner, W., *Australian J. Exp. Biol. Med. Sci.*, 1940, **18**, 253.

8 Boor, A. K., and Miller, C. P., *J. Exp. Med.*, 1934, **59**, 63.

9 Boor, A. K., and Miller, C. P., *Arch. Path.*, 1941, **29**, 724.

10 Boor, A. K., and Miller, C. P., *J. Inf. Diseases*, 1944, **75**, 47.

11 Miller, C. P., Becker, R. M., Schad, Doretta, and Robbins, M. Wright, *J. Inf. Dis.*, 1943, **73**, 248.

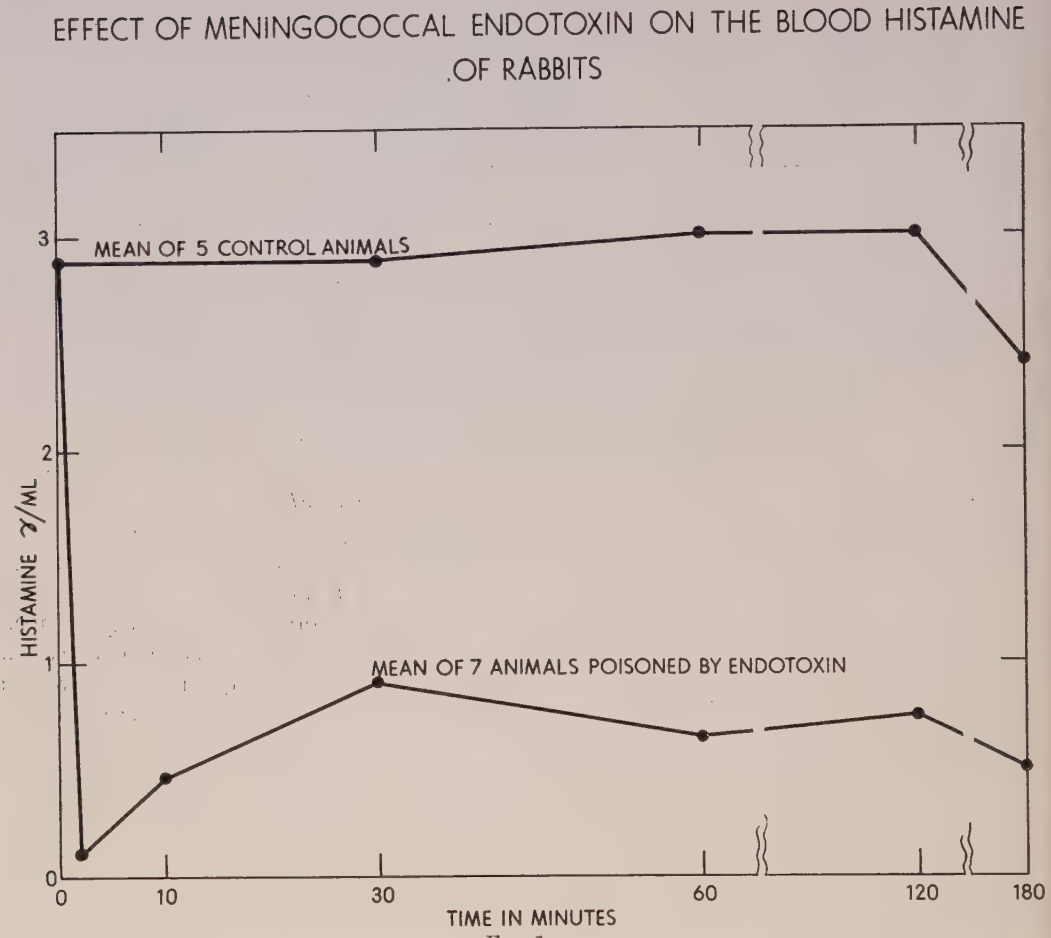

FIG. 1.

Thirty-eight rabbits, weighing from 2.4 to 3.7 kg were used as experimental animals. No anesthesia was used, because it was found that narcosis itself caused significant changes in the blood histamine content of the rabbit.† Blood was removed by cardiac puncture and immediately delivered into a weighed amount of 10% trichloracetic acid. The extraction was made shortly afterwards. Blood histamine was determined according to the method of Barsoum and Gaddum,[12] using the modification suggested by Code.[13] The histamine content of the tissues was determined on weighed samples ground in a glass tissue homogenizer[14] in 10% HCl. Lipoids were removed from the tissue homogenates by ether extraction. The isolated ileum of the guinea pig, suspended in atropinized Tyrode at 37°C, was used as test object and the height of contraction was read directly on a scale. Each figure given in the tables and graphs is the mean of 5 parallel measurements (the S.D. did not exceed ±15%). In all measurements, the effect of known amounts of histamine phosphate was compared with the tissue extracts, and the results were expressed in terms of histamine base.

Results. Control Observations. In 5 normal rabbits the histamine content of the blood was determined at 0, 30, 60, 120, and 180 minutes. There was no significant variation in the blood histamine level during the course of

† Unpublished experiments.

[12] Barsoum, G. S., and Gaddum, G. H., *J. Physiol.*, 1938, **85**, 1.

[13] Code, C. F., *J. Physiol.*, 1937, **89**, 257.

BLOOD HISTAMINE OF RABBITS AFTER INTRAVENOUS INJECTION OF HISTAMINE

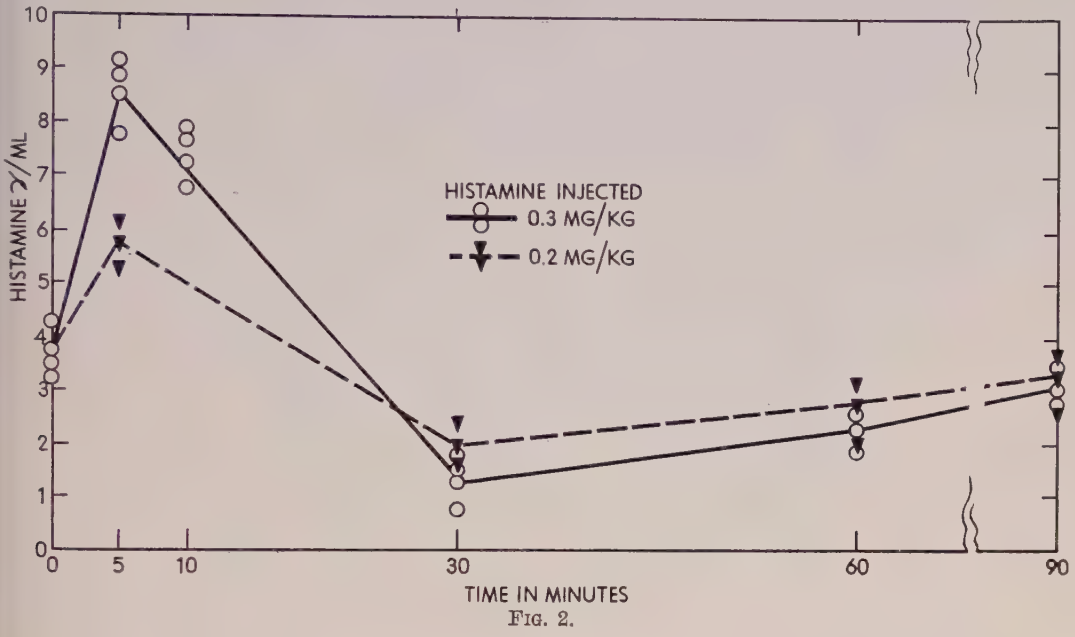

FIG. 2.

the experiment.

In the first series of experiments the concentration of histamine was studied in the blood of 7 rabbits, following the intravenous injection of 3.6 ml of endotoxin per kg body weight.

In all experiments the histamine content of the blood fell sharply immediately after the intravenous injection of endotoxin and remained well below its normal level as long as the animal lived—a period of 2-3 hours. (Fig. 1)

The mechanism involved in the disappearance of the blood histamine seems to be rapid. Since the endotoxin was the only foreign substance introduced into the animal body in these experiments, it might be supposed that endotoxin inactivates histamine. It was found, however, that when various amounts of histamine phosphate were added to the toxin, they could be recovered without significant loss by the same extraction method used for the blood analyses.

In the second series of experiments, the effects of simultaneous injections of histamine and endotoxin were studied in 7 rabbits. In each experiment a control observation was first made by injecting histamine alone. When 0.3 mg histamine per kg body weight, in the form of histamine phosphate, was injected intravenously, the blood histamine content rose in 5 minutes to a level 2 to 3 times the normal. A 50% rise in blood histamine level was caused by an injection of 0.2 mg histamine per kg. (Fig. 2)

When meningococcal endotoxin (3.6 or 1.8 ml per kg) was injected simultaneously with a dose of histamine, which varied from 0.3 to 0.1 mg per kg no increase in blood histamine occurred. In each instance it fell to a significant degree. (Fig. 3)

The simultaneous injection of meningococcal endotoxin plus histamine markedly shortened the survival time of the rabbits. A dose of endotoxin, which usually caused death in 7-10 hours (1.8 ml toxin per kg), when injected simultaneously with 0.3-0.15 mg histamine per kg killed the animal in 5-10 minutes. These amounts of histamine alone injected intravenously were never fatal.

In the third series of experiments, determinations were made of the histamine con-

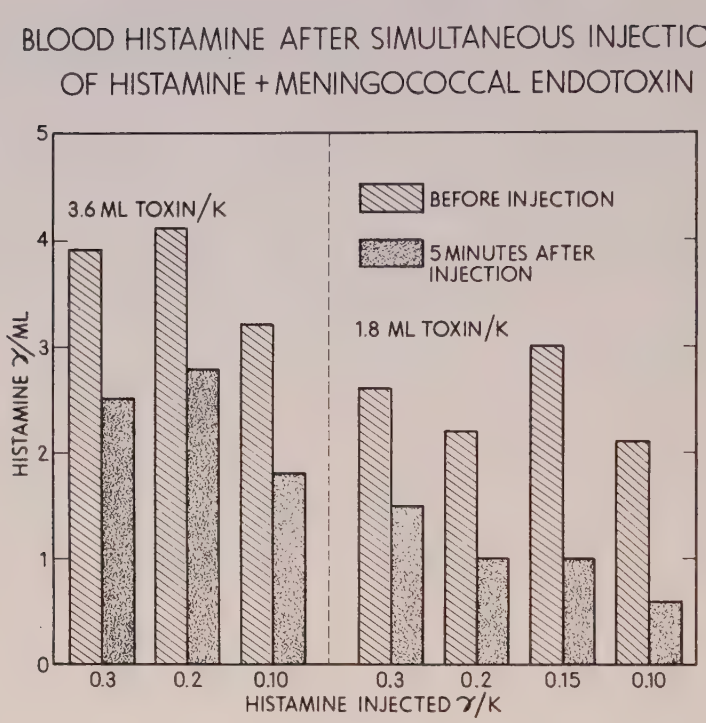

FIG. 3.

tent of the tissues of rabbits poisoned by meningococcal endotoxin. Seven animals were injected intravenously with 3.6 ml meningococcal endotoxin per kg, and their tissues analyzed immediately after death. The results are given in Fig. 4, which includes, for comparison, the histamine content of the organs of 5 normal rabbits. A significant increase in muscle and liver histamine was observed. The histamine content of the liver, as well as that of the diaphragm, showed about a 4-fold increase over the normal, while the skeletal muscle showed an average increase of 2.4 times the normal.

Discussion. These experiments show that the intravenous injection of meningococcal endotoxin into rabbits is followed by a rapid decrease in the histamine content of the blood and a considerable increase in the histamine content of the liver and muscle. The decrease in histamine content of the blood amounted to 2.75 histamine per ml of blood. If one assumes the total blood volume of a 3 kg rabbit to be 7% of its body weight[14] the total quantity of histamine which disappeared from the blood was 0.77 mg; *i.e.*, 0.26 mg per kg. Based on the results of Levin and co-workers[15] who determined the average weight of rabbit organs, the amount of histamine accumulated in the tissues was about 5 mg. (1.7 mg histamine per kg in a 3 kg rabbit.) Since the amount of histamine which disappeared from the blood is about 1/7 the amount found in the tissues of poisoned animals, it is probable that most of the increased tissue histamine did not originate from the blood but was formed in the tissues themselves. The tissues of the poisoned rabbits contained about 3 times more histamine than the lethal intravenous dose of this substance[16] which circumstance suggests that histamine may be responsible for the death of the animals. These experiments, however, do not provide con-

[14] Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **116**, 495.

[15] Levine, C. J., Mann, W., Hodge, H. C., Ariel, I., and DuPont, O., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 318.

[16] Sollmann, T., and Hanzlik, P. J., *Introd. to Exp. Pharmacol.*, 1928, p. 284.

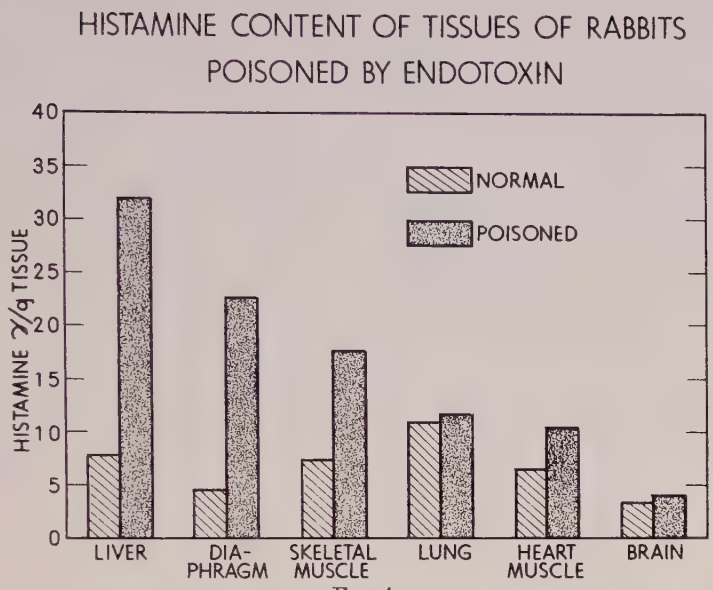

FIG. 4.

clusive proof of this supposition.

Summary. Meningococcal endotoxin injected intravenously into rabbits caused a rapid decrease in the histamine content of the blood and an increase in the histamine content of liver and muscle.

The decrease in histamine in the blood was not prevented by the intravenous injection of histamine along with the endotoxin.

The survival time of rabbits injected intravenously with meningococcal endotoxin was markedly shortened by a simultaneous injection of a small dose of histamine.

The author wishes to take this opportunity to thank Dr. C. Phillip Miller and Dr. Alden K. Boor of the Department of Medicine for supplying the meningococcal endotoxin and for their advice regarding these experiments.

16035

Effect of Oral Streptomycin on the Intestinal Flora.

L. W. KANE AND G. E. FOLEY, (Introduced by L. Dienes.)

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The majority of evidence seems to indicate that *E. coli* plays a predominant role in the pathogenesis of peritonitis following large bowel surgery.[1] Among the other microorganisms most frequently encountered are the fecal streptococci (Lancefield Group D streptococci), and *Clostridium welchii*.[1] With these facts in mind, it seems logical to suppose that the incidence of post-operative peritonitis would be reduced if the *E. coli* present in the bowel were eliminated. The less readily absorbable sulfonamides such as sulfaguanadine, sulfasuxidine and sulfathaladine have been used in an endeavour to accomplish this.

1 Meleney, F. L., Harney, H. D., and Jern, H. Q., *Arch. Surg.*, 1931, **22**, 1.

Streptomycin as an antibacterial agent in the intestine, has the following properties: (1) soluble in water, (2) non-absorbable through the intestine, (3) non-toxic, (4) active in the presence of intestinal contents. Six cases were selected for study. Three of these had ulcerative colitis and 2 were pre-operative cases—a uretero-enterostomy and a large bowel resection. One was a normal individual. The 2 cases of ulcerative colitis, one pre-operative large bowel resection, and one normal individual were each administered 1.0 g of streptomycin *per os* daily, in 2 divided doses (0.5 g dissolved in a half-glass of water, morning and evening). The pre-operative uretero-enterostomy received 0.4 g of streptomycin *per os* 5 times daily. The third case of ulcerative colitis received streptomycin as a lavage per rectum; 20 ml containing 0.005 g streptomycin per ml was administered twice daily in an attempt to rid the distal segment of an old colostomy of *E. coli*.

Aerobic and anaerobic cultures were planted daily with a generous amount (approximately 0.5 g) of fresh stool collected from each patient. Bacterial counts per gram of wet stool were estimated from duplicate horse blood agar plates streaked with 0.05 ml of a 1:1,000,000 dilution of stool. In the 2 pre-operative cases who received streptomycin, swabs taken directly from the mucosa of the colon at operation were planted in aerobic and anaerobic cultures.

The results obtained in all 6 cases were essentially similar. A representative case is summarized in Table I. As can be seen in the Table, *E. coli* disappeared from the stool after streptomycin had been administered for 2 days. In one case, a normal individual, *E. coli* disappeared in one day, and in the remaining 4 cases, these microorganisms could not be cultured after 2 days on streptomycin.

Since microscopic examination of the stool showed an abundance of Gram negative rods even though *E. coli* could not be cultivated in aerobic or anaerobic media, daily motility tests were done on freshly collected stool specimens in order to ascertain whether or not these microorganisms actually were dead. In

TABLE I.

Effect of Oral Streptomycin on the Intestinal Flora.

Day of study	G streptomycin	Stool examination								
		Microscopic		Cultural—Aerobic and anaerobic						Aerobic colony count*
		Bacteria	Motility	*E. coli*	Streptococci	Bacteroides	Clostridia	Candida	
0	0	++++	++	++	++++	++++	++++	0	—
1	1.0	++++	++	++++	++++	++++	++++	0	52.4†
2	1.0	++++	±	++	++++	++++	++++	0	6.6‡
3	1.0	++++	0	0	++++	++++	++++	++++	0
4	0.5	++++	0	0	++++	++++	++++	++++	—
5	0	++++	0	±	++++	++++	++++	++++	—
6	0	++++	++++	++++	++++	++++	++++	++++	—

* Per g of wet stool in billions.
† *E. coli* predominant.
‡ Streptococci predominant.

all cases, when *E. coli* could no longer be cultivated from the stool, wet smears made directly from the specimen failed to show motile microorganisms. The failure of *E. coli* to grow in the presence of sodium thioglycollate or under anaerobic conditions, both of which have been reported as opposing the *in vitro* action of streptomycin,[2,3] is further evidence that these microorganisms were nonviable. Since Bacteroides, a common anaerobic saprophyte of the large bowel could be cultivated from most of these cases, even after *E. coli* had disappeared, it is possible that the Gram negative, non-motile, rods observed by direct microscopic examination belonged to this genus.

E. coli reappeared in the stool the day following the last dose of streptomycin. Fecal streptococci, Clostridia, Bacteroides and Candida were unaffected by the streptomycin in the stool. It is noteworthy that Candida appeared in the stool on occasions after *E. coli* had disappeared (Table I). No change in the character of the stool was observed during streptomycin administration. The color, consistency and quantity was the same during therapy as it had been before.

Discussion. The results obtained in this study are similar to those reported by Reimann, Price and Elias,[4] and others,[8] who found that sensitive microorganisms could be eliminated from the stool by oral administration of streptomycin; and that the stool could be kept free of such microorganisms as long as adequate streptomycin levels were maintained. These authors also noted that the anaerobic flora of the stool was unaffected by oral streptomycin.

It is of interest that although *E. coli* was eliminated from the stool in 3 cases of ulcerative colitis, no apparent effect upon the course of the disease was noted. In order to determine whether or not viable *E. coli* were present on the surface of the mucosa of the colon, in the cases who received streptomycin preoperatively, direct swabs were taken at operation. In neither of these cases could *E. coli* be cultivated.

It is known that the non-absorbable sulfonamides will reduce the number of *E. coli* present in the stool. In some cases, however, no reduction has been observed, yet in spite of the failure of these drugs to eliminate *E. coli*, clinical reports indicate that pre-operative administration decreases the incidence of complicating peritonitis and abscess formation following surgical manipulation of the colon.[5,6,7] It would seem on the basis of this study, that streptomycin is more effective than the sulfonamides in ridding the colon of *E. coli*. It has the further advantage of avoiding the risks associated with the use of the sulfonamides. Accordingly, the oral administration of streptomycin in the preoperative preparation of patients who are to undergo surgery of the colon is suggested.

Summary. The oral administration of as little as 1.0 g of streptomycin daily eliminated *E. coli* from the stool of 5 patients within 2 days. The stool could be kept free of these microorganisms as long as adequate streptomycin levels were maintained, but reappeared promptly when it was discontinued. Swabs taken at operation from the mucosa of the colons of two patients who had received preoperative streptomycin did not contain *E. coli*. These microorganisms also were eliminated from the distal segment of a colostomy by streptomycin lavage per rectum. Streptomycin did not affect the anaerobic flora of the stool, and had no appreciable effect on the fecal streptococci.

2 Donovick, R., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 224.

3 Geiger, W. B., Green, S. R., and Waksman, S. A., PROC. SOC. EXP. BIOL., AND MED., 1946, **61**, 187.

4 Reimann, H. A., Price, A. H., and Elias, W. F., *Arch. Int. Med.*, 1945, **76**, 269.

5 Zintal, H., Lockwood, J. S., and Snyder, J., *Bull. Am. Coll. Surg.*, 1943, **28**, 51.

6 Behrend, M., *Surg. Clin. North Am.*, 1944, **24**, 238.

7 Bacon, H. E., *et al.*, *J. Internat. Coll. Surg.*, 1945, **8**, 20.

8 Pulaski, E. J., and Amspacher, W. H., *Bull. U. S. Army M. Dept.*, 1946, **6**, 750.

16036 P

Effect of Penicillin on the Reaction Between Phage and Staphylococci.

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In Fleming's original paper on penicillin,[1] he described the lysis of staphylococci by this agent. Since then the lytic process and ancillary morphologic changes in the bacteria concerned have been studied by several investigators.[2-8] We wish to report here the effect of penicillin on the lytic action of bacteriophage.*

The phage employed in our experiments is the "K" race; it and the homologous strain of *Staphylococcus aureus* have been used in studies described in a series of papers appearing since 1929. Stock cultures were grown in Roux flasks for 18 hours at 36°C. In order to have a substrate of actively growing cells, 1 x 10^8 staphylococci/ml were suspended in 100 ml of broth contained in a 500 ml flask; aeration was provided by placing the flask in a shaker operating in a water bath set at 36°C. When growth had progressed to a level of 1 x 10^9 bacteria/ml, the cell suspension was removed from the shaker and used for the experiments described below. To determine [bacteria] when values were >5 x 10^8 staphylococci/ml, the direct microscopic count method was employed. For lytic curves, visual comparison was made with standards covering a range from 2 x 10^7 to 5 x 10^8 staphylococci/ml in formalinized broth. The fluid medium throughout was tryptose phosphate broth. Phage determinations were carried out by Gratia's method[9] of counting plaques and the values noted below are in plaques/ml.

A 10 ml mixture containing 5 x 10^8 staphylococci/ml, 5 x 10^8 phage units/ml and 10 units of sodium penicillin/ml was placed in a test tube and shaken at 36°C. Turbidity readings were made every 0.2 hour. This was also done with a suspension from which the phage was omitted and with one containing no penicillin. Using as an end-point the reduction of turbidity by 50%, *i.e.*, to 2.5 x 10^8 staphylococci/ml, it was observed that the suspensions lysed in the following order: Phage + penicillin, 0.8 hours; phage only, 2.0 hours; penicillin only, 2.7 hours.

When the initial concentration of phage was reduced to 5 x 10^7 phage units/ml, the respective times of lysis were: Phage + penicillin, 1.1 hours; phage only, 3.8 hours.

The accelerating effect could not be demonstrated in suspensions made up in Locke's solution; lysis was considerably delayed in the phage + penicillin suspension and the lytic curve paralleled that for penicillin alone.

Repetition of the experiment with concentrations of penicillin varying from one unit/ml to 1,000 units/ml showed that the acceleration of lysis took place uniformly in all concentrations and was independent of (penicillin) within this range, 0.01 unit of penicillin/ml and lesser concentrations had no measurable effect in speeding up phage-engendered lysis.

An experiment was performed to determine how long a period of penicillin action was required in a mixture of bacteria and phage

1 Fleming, A., *Brit. J. Exp. Path.*, 1929, **10**, 1.

* Since preparation of this article for publication, Dr. Winston Price of Rockefeller Institute has informed us in a personal communication of similar studies he has completed using another phage and bacterial substrate.

2 Gardner, A. D., *Nature*, 1940, **146**, 837.

3 Fleming, A., *Lancet*, 1941, **241**, 761.

4 Smith, L. D., and Hay, T., *J. Franklin Inst.*, 1942, **233**, 598.

5 Weiss, L. J., *Proc. Indiana Acad. Sci.*, 1943, **52**, 27.

6 Miller, C. P., and Foster, A. Z., Proc. Soc. Exp. Biol. and Med., 1944, **56**, 205.

7 Todd, E. W., *Lancet*, 1945, **248**, 74.

8 Fisher, A. M., *J. Bact.*, 1946, **52**, 539.

9 Gratia, A., *Ann. Inst. Pasteur*, 1936, **57**, 652.

to produce the characteristic acceleration. Several 9 ml mixtures of phage and bacteria in test tubes were prepared containing 5 x 10^8 staphylococci/ml and 2.5 x 10^8 phage units/ml. The tubes were placed in the shaker at 36°C and at intervals of 10, 20, 40, and 60 minutes from the time of mixing, one ml of penicillin solution (10 units) was added to successive tubes.

The addition of penicillin at 10 minutes and 20 minutes produced 50% lysis 0.7 hours ahead of the tube containing phage only. When penicillin was added after 40 minutes had elapsed, the lytic end-point occurred 0.5 hours before that in the phage control. With these particular concentrations of bacteria and phage, a typical acceleration could be secured if the penicillin acted at least 0.9 hours on the cellular substrate. Exposure to penicillin for a period of from 0.4 to 0.7 hours reduced the time of lysis by 0.5 hours.

In many of the experiments performed when the initial concentrations of bacteria were 5 x 10^8 staphylococci/ml or greater, the initial phage concentration 5 x 10^8 phage units/ml and the concentration of penicillin between 10 and 1,000 units/ml, clearing of the suspensions occurred without any increase in turbidity. Despite the absence of obvious bacterial reproduction, there were increases in [phage], up to a maximum of 10-fold, as determined by the plaque count. The methods used for detecting any increase in [bacteria] were not critical, however, and this point should be reinvestigated with more sensitive procedures.

A detailed account of further experiments with penicillin and phage action will be published elsewhere.

16037

A Bacteriophage for *Mycobacterium smegmatis.**

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During investigations on the isolation of microorganisms antagonistic to the mycobacteria a bacteriophage active for *Mycobacterium smegmatis* was encountered.[1] The finding of this bacteriophage is of interest because it is, to our knowledge, the first definite evidence of a bacteriophage among the mycobacteria. Steenken[2] described spontaneous lysis in old colonies of *Mycobacterium tuberculosis* H37Rv which may have been due to bacteriophage action. He determined that the lytic factor was filterable but did not identify it as a bacteriophage by all of the usual criteria. Lysis appeared when the cultures were 3 to 4 months old and the pH of the medium had dropped to values of about 4.2 to 4.6. It began in the center of the colonies and spread to the periphery. The filtrate produced lysis of living bacteria at pH 4.2 to 4.8 and of both living and heat-killed bacteria at pH 2.2 to 4.0. Secondary resistant colonies developed in old liquefied areas.

The fact that bacteriophages for the mycobacteria have not been reported previously suggests that they may be very limited in their distribution. The successful isolation of one of them in the present work may have been due to the particular enrichment treatment employed.

The enrichment was carried out on 200-g samples of moist leaf compost to which a small amount of calcium carbonate was added. The

* This work was supported by a grant from the Alice McDermott Research Foundation of the University of Washington.

1 Weiser, R. S., and Gardner, G. M., unpublished results.

2 Steenken, W., *Am. Rev. Tub.*, 1938, **38**, 777.

samples were collected from various locations in the city. They were incubated at 37°C for 8 months and treated semi-weekly with 5 to 10 ml of a heavy well-washed suspension of a young culture of *M. smegmatis*. The suspension was prepared from organisms grown on glycerine broth for one week at 37°C. The culture mass was ground in a mortar and washed twice in physiological salt solution.

After 3 months of enrichment, tests for organisms antagonistic for *M. smegmatis* were begun by making fixation plates of dilutions of the compost ranging from 1:100 to 1:-100,000. The medium used for plating was nutrient agar containing 1% glycerine and heavily inoculated with *M. smegmatis*.

Soon after beginning the tests, bacteriophage plaques were noted on the plates made from two of the compost samples. They had smooth edges and displayed a halo of partial lysis about a central clear zone of complete lysis. Isolated plaques attained a diameter of about 3 mm. Smears made from the clear areas contained only an occasional acid-fast organism. The material from the clear areas, when sub-cultured on bacteriological media, likewise, yielded only acid-fast organisms.

A Berkefeld filtrate was prepared from plaque material and the bacteriophage subcultured several times on plates. Filtrates of plaque material from the sub-cultures contained the bacteriophage in a concentration of 300 billion particles per ml.

No particular attempt was made to determine that the bacteriophage was a pure strain. However, preliminary tests indicate that the bacteriophage is specific for our stock strain of *M. smegmatis*. It proved to be inactive for *Mycobacterium phlei* and a second strain of *M. smegmatis*.

The bacteriophage was inactivated by a temperature of 75°C for 10 minutes but remained active when held at 72°C for 10 minutes. It preserved well in 50% glycerine and by lyophilization.

As a test of the effectiveness of the method used for the isolation of bacteriophage for *M. smegmatis* the enrichment procedure was repeated using a sample of soil collected on the University campus. Precautions were taken in this trial to prevent possible contamination of the test plates with the specific bacteriophage we had previously isolated. Weekly tests for the presence of bacteriophage were negative until the third week of enrichment when plaques first appeared. Tests on the control unenriched sample of soil were negative for bacteriophage. The plaque characteristics of this newly isolated bacteriophage did not appear to be different from those of the previous isolate.

Discussion. In our work on the isolation of microorganisms antagonistic to the mycobacteria we have used both enriched and unenriched soil and compost. The bacteriophage was isolated from 2 of 6 samples of enriched compost and from one sample of enriched soil. It was not encountered in 8 samples of unenriched compost and 4 samples of soil. Apparently the isolation of the bacteriophage was facilitated by the specific enrichment employed, and possibly because the enrichment was carried out on the soil and compost samples preliminary to filtration rather than after filtration.

Bottcher and Hofer[3] have employed specific preliminary enrichment of soil for the isolation of bacteriophage for the legume bacteria by adding a special medium heavily inoculated with the organism. The present method differs from that of Bottcher and Hofer inasmuch as the organisms added were free of medium. It is possible that the lack of medium may operate to advantage by reducing the possibility of suppression of the specific organism or its bacteriophage by overgrowth of some other organism.

Summary. A bacteriophage specific for *M. smegmatis* was isolated from samples of compost and soil by specific enrichment with a heavy washed suspension of *M. smegmatis*. The method of enrichment employed may be useful in the isolation of other phages.

[3] Bottcher, J., and Hofer, A. W., *J. Bact.*, 1943, **45**, 407.

16038

Tissue Toxicity of the Germicides Iodine and Bromine.*

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Lambert[1,2] as early as 1916, defined the ideal antiseptic as one which kills the infecting agent without causing injury to body cells. In accordance with this definition he evaluated a series of germicides in terms of relative toxicity to *Staphylococcus aureus* and human connective tissue cells respectively. In different later investigations, various other tissues were used for tests of this type. Lampert and Meyer[3] used rabbit spleen, German[4] and Buchsbaum and Bloom[5] used chick tissue culture, while Salle and Lazarus[6] used embryonic chick heart tissue for their experiments. In experiments carried out by Salle and McOmie[7] and Salle, McOmie and Schechmeister,[8] embryonic tissue was used as the test substance. Similar investigations were carried out by Osgood[9] and Herrell and Heilman.[10]

In other methods of determining toxicity for tissue, living chick embryos have been employed as the test object. The living embryo is preferable to tissue culture for this purpose as it presents conditions which approach more closely the complexity of the tissue interrelations of the intact animal. The developing egg presents the additional advantage that it consists of rapidly growing embryonic tissue in a perfect nutritional environment, and furthermore contains large amounts of albuminous material with which the bactericidal agent can come into contact. Moreover, the chick embryo is convenient to handle because of mechanical protection and the ease of repeated access to the embryo which is afforded by the egg shell.

Tests of disinfectants for their potency *in vivo* by chick embryo methods, were carried out by Witlin,[11] Dunham,[12] and Green and Birkeland.[13]

In a third method the toxicity test is carried out in the presence of blood. The use of this test object is indicated because of its known role in infection, and because of the high sensitivity of blood cells to the germicides. Welch and Brewer[14] claim that "application of antiseptics which destroy this function at dilutions which cannot destroy bacteria is a harmful practice". In papers by Nye,[15] Welch and Hunter,[16] and Hirsh and Novak,[17] phagocytes were used as test object of the toxicity assays.

In the following experiments the two last

* Based on data submitted in partial fulfillment of the requirement for the degree of Ph.D., The Hebrew University, January, 1946.

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1 Lambert, R. A., *J. Exp. Med.*, 1916, **24**, 683.

2 Lambert, R. A., *J. A. M. A.*, 1916, **67**, 1300.

3 Lambert, R. A., and Meyer, J. R., Proc. Soc. Exp. Biol. and Med., 1926, **23**, 429.

4 German, W. J., *Arch. Surg.*, 1929, **18**, 1920.

5 Buchsbaum, R., and Bloom, W., Proc. Soc. Exp. Biol. and Med., 1931, **28**, 1060.

6 Salle, A. J., and Lazarus, A. S., Proc. Soc. Exp. Biol. and Med., 1935, **32**, 1481; **33**, 8, 665, 937, 1057, 1119.

7 Salle, A. J., McOmie, W. A., and Schechmeister, I. L., *J. Bact.*, 1937, **34**, 267.

8 Salle, A. J., McOmie, W. A., and Schechmeister, I. L., Proc. Soc. Exp. Biol. and Med., 1938, **37**, 694.

9 Osgood, E. E., *Arch. Int. Med.*, 1938, **62**, 181.

10 Herell, W. E., and Heilman, D., *Am. J. Med. Sci.*, 1943, **205**, 157.

11 Witlin, B., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 27.

12 Dunham, W. B., Proc. Soc. Exp. Biol. and Med., 1942, **50**, 274.

13 Green, T. W., and Birkeland, J. M., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 55.

14 Welch, H., and Brewer, C. M., *J. Immunol.*, 1942, **43**, 25.

15 Nye, R. N., *J. A. M. A.*, 1937, **108**, 280.

16 Welch, H., and Hunter, A. C., *Am. J. Pub. Health*, 1940, **30**, 129.

17 Hirsch, M. M., and Novak, M. V., Proc. Soc. Exp. Biol. and Med., 1942, **50**, 376.

TABLE I.
Effect of Halogens on Phagocytosis.

Exp. No.	Halogen	Concentration, %	Polynuclears taking part in phagocytosis, %	No. of bacteria ingested per 100 cells
1	Br_2	1.75	45.7	1.2
	I_2	0.5	13.5	0.27
2	Br_2	1.25	52.0	1.76
	I_2	0.25	25.5	0.57

mentioned methods were used with some modifications.

In earlier tests comparing the disinfectant activities of bromine and iodine, it was observed that bromine *in vivo* (rabbit test) was more active than iodine, whereas the latter was more effective than bromine as a disinfectant *in vitro* (Rideal-Walker test). This suggested that bromine applied on living tissue might be a more efficient antiseptic for use on wounds, cuts and abrasions than iodine.

Toxicity tests on both halogens were undertaken. The method of Hirsh and Novak[17] was employed. In this method the highest dilution which destroys the phagocytic power of leucocytes is determined (toxicity end point) and divided by the highest dilution which kills the test organism (germicidal end point) under the same conditions. Thus, by dividing both end points a toxicity index which expresses the disinfectant power of the tested substance *in vivo* is obtained. A difficulty was encountered in carrying out the test as described by Hirsh and Novak.[17] Even when 7 parts of saturated bromine solution of 3.5% were added to one part of blood marked phagocytosis still occurred. Thus, when the prescribed amount of blood was decreased from 50% to 12.5%, the toxicity end point was still not reached. It is clear, however, that the toxicity index is well under 1, and therefore highly favorable.

In another series of experiments a slight modification of this experimental technique was employed. The germicidal end point was determined as described above, but the harmful effect of the halogens on the leucocytes was evaluated by making blood smears after contact of the blood cells with the halogens for 30 minutes at 37°C in dilution which prevents growth of the test organism. The bacteria were added and a contact time of 10 minutes was allowed, the mixture being shaken thoroughly in the meantime in a water bath of 37°C to assure contact between blood cells and bacteria. After staining the blood smears with Loeffler's methylen blue, 100 polymorphonuclear leucocytes were counted and (1) the number of the cells taking part in phagocytosis and (2) the number of bacteria ingested were determined. By comparing the effect of the 2 halogens, the one more harmful to the leucocytes could be recognized. The results are summarized in Table I.

In a further series of experiments, a modification of Green and Birkeland's[13] method for testing a disinfectant on living embryo was used. Chorio allantois of living embryos was infected with *S. aureus* according to Goodpasture and Buddingh.[18] The amount of bacteria was so chosen that the embryo was killed after 24-48 hours. When disinfectant is added in an appropriate amount, it stops the development of the bacteria, and the embryo can survive the infection. The disinfectant to be tested is used in a concentration which in blank experiments does not have a harmful effect on the embryo. After infecting the chorio allantois with a lethal dose of bacteria, a specific time is allowed to enable the bacteria to begin logarithmic growth. The proper time was determined by agar plate counts. The disinfectant to be tested is added about 3-4 times during 48 hours. By comparing the death rate occurring after treatment with various disinfectants, the relative efficacies were determined.

[18] Goodpasture, E. W., and Buddingh, G. J., *Am. J. Hyg.*, 1935, **21**, 319.

TABLE II.
Antibacterial and Toxic Action of Halogens on Living Chick Embryos.

Bacteria inoculated	Halogen	Conc.,%	No. of eggs under exper.	No. of dead embryos after 48 hr	Death rate, %
$2x10^6$	—	—	23	20	87
$2x10^6$	Br_2	0.2	11	5	45
$2x10^6$	Br_2	0.06	12	6	50
$2x10^6$	I_2	0.08	16	14	87.5
—	I_2	0.08	5	1	20
—	Br_2	0.1	5	0	0
—	Br_2	0.06	5	1	20

In each experiment $2x10^6$ cells of *S. aureus* from 18-24-hours-old broth culture were used. The eggs were placed for 2-3 hours in an incubator at 37°C to allow the bacteria to begin to multiply. The disinfectants were added after 3, 8, and 24 hours. In control experiments saline instead of halogens was added. The embryos were opened after 48 hours. The results obtained with bromine and iodine are given in Table II. These experiments, too, show that bromine in presence of living tissue is a better disinfectant than iodine.

Conclusion. The assertions of Babcock[19] concerning the effectiveness of bromine *in vivo* in treatment of wounds is confirmed by the toxicity tests.

[19] Babcock, W. W., *J. A. M. A.*, 1945, **129**, 1094.

The author wishes to state that this investigation owes much to the interest and help of her late teacher, Prof. I. J. Kligler.

16039

Mumps Meningo-Encephalitis. Isolation in Chick Embryos of Virus from Spinal Fluid of a Patient.*

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Meningo-encephalitis may occur as a complication of parotitis or as a primary manifestation of infection with the virus of mumps. Whereas in the first instance the time relationships tend to permit a diagnosis of mumps meningo-encephalitis, the diagnosis of the primary encephalitic manifestation has been placed on a firm basis only since the development of a complement-fixation technic by Enders and his co-workers.[1] The demonstration of a rise in complement-fixing antibodies in convalescent serum as compared to the antibody titer in the serum specimen obtained in the first days of the disease may be taken as diagnostic evidence of mumps.[2] By this means, a considerable number of cases of meningo-encephalitis without prior or concomitant parotitis can be diagnosed as due to mumps.[3] Differentiation between antibodies to the soluble and to the virus antigens[4] may be of further diagnostic help.

* The work described in this paper has been aided by the Office of Naval Research.

[1] Enders, J. F., Kane, L. W., Cohen, S., and Levens, J. H., *J. Exp. Med.*, 1945, **81**, 93.

[2] Enders, J. F., Cohen, S., and Kane, L. W., *J. Exp. Med.*, 1945, **81**, 119.

[3] Kane, L. W., and Enders, J. F., *J. Exp. Med.*, 1945, **81**, 137.

[4] Henle, G., Henle, W., and Harris, S., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 290.

Antibodies to the soluble antigen tend to appear earlier than antibodies to the virus antigen in a high percentage of infections with the mumps virus. When this pattern of serum reactivity occurs, it is only found in the first few days of infection.[5]

Another approach to the diagnosis of mumps meningo-encephalitis has been the attempt to isolate the etiological agent from the spinal fluid. One such attempt has been successful in the monkey[6] but inoculation of chick embryos failed.[6,7] In the case to be reported here a virus was obtained from the spinal fluid of a patient with meningo-encephalitis without parotitis on the second day of illness by the intra-amniotic inoculation of chick embryos. This agent has been identified as mumps virus.

Case history. The 5½-year-old patient A.P. was admitted to the hospital on 17 March 1947, complaining of fever, severe headache, and occasional vomiting of 2 days duration. The child appeared moderately ill. Physical examination revealed mild nuchal rigidity but no other neurological signs. A slight injection of the pharynx and of the orifices of Stenson's ducts was noted but no parotid or other glandular swelling. The temperature on admission was 103°F, the pulse 108 per minute. All other examinations were negative.

In the night following admission the temperature rose to 105°F, but thereafter decreased steadily and returned to normal on the 4th day in the hospital. The headache and nuchal rigidity persisted for 2 days. On the day after admission, 18 March 1947, a slight disturbance in swallowing was reported which was of very short duration, and complaints of moderate abdominal pain were reported. Thereafter the child improved steadily and was released from the hospital after 15 days on 1 April 1947. There was no known exposure of the patient to mumps. However, a sister developed parotitis on 29 March 1947.

Laboratory findings. Spinal fluid was obtained from the patient on the day of admission, *i.e.,* on the second day of illness. The initial pressure was 450 mm H_2O, the final recording 290 mm. The fluid was slightly cloudy and contained 450 cells per cu mm, of which 92% consisted of lymphocytes, 7% of endothelial cells, and 1% of polymorphonuclear leukocytes. No bacteria could be demonstrated in stained smears, nor could any organisms be cultured on bacteriological media. The total protein in the fluid was 10 mg, sugar, 30 mg and chlorides 712 mg/100 ml. Subsequent spinal fluids taken 24 March and 31 March 1947 showed similar, although increasingly milder, changes, the last specimen containing 55 cells per cu mm.

The blood picture on admission was as follows: 4,500,000 erythrocytes, 13 g hemoglobin, 17,000 white cells, of which 60% were granulocytes, 40% lymphocytes, and 4% monocytes. A subsequent study of the blood on 31 March 1947 was essentially normal.

Other studies included tuberculin tests with O.T. 1:10,000 and 1:1,000, which were negative. The Kolmer-Wassermann, Kahn and complement-fixation tests for lymphocytic choriomeningitis with the serum of the patient taken 1 April 1947, likewise, were negative. Attempts to isolate this virus from the spinal fluid drawn 17 March 1947 failed.†

Serological tests for mumps. The preparation of complement-fixation antigens and the technic used for the complement-fixation test have been described.[4] The tests performed with the sera obtained during the early stage of the disease were not revealing in this case.

TABLE I.
Result of Complement Fixation Tests with Sera from Patient A.P.

Serum specimen	Day after onset of disease	Serum titer (initial dilution) vs. Soluble antigen	Virus antigen
3-17-47	2	1: 4*	1: 4
21	6	1: 4	1: 8
25	10	1: 8	1: 32
4- 1	17	1:16	1:128

* Complete fixation of complement in serum dilution 1:4.

† These tests were performed by Dr. M. M. Sigel.

5 Henle, G., to be published.

6 Swan, C., and Mawson, J., *Med. J. Austral.,* 1943, **1**, 411.

7 Beveridge, W. I. B., Lind, P. E., and Anderson, S. G., *Austral. J. Exp. Biol. and Med. Sci.,* 1946, **24**, 15.

As can be seen in Table I, low concentrations of antibodies to the soluble as well as to the virus antigen were found on the 2nd day after onset. However, a definite diagnosis of infection with mumps virus was possible because subsequent specimens of serum showed significant rises in antibodies to both antigens.

Isolation of virus. Spinal fluid, drawn on the second day of illness was kept frozen at −10°C until injection of chick embryos was possible. Eight 8-day-old embryos were inoculated into the amniotic sac with 0.1 ml of undiluted spinal fluid each. After incubation of the eggs at 36 to 37°C for 5 days, the amniotic and allantoic fluids of each embryo were collected separately and tested on a slide for their capacity to agglutinate chicken red cells.[8] A slight degree of hemagglutination was observed with several of the amniotic fluids. The bacteriologically sterile pool of all amniotic fluids was used for further passage. Five out of 16 embryos of the second amniotic passage died, and of the 11 remaining embryos 10 amniotic fluids but none of the allantoic fluids showed positive hemagglutination. On the 4th amniotic passage the virus in the amniotic fluid reached a 50%-infectivity end point of $10^{-6.3}$ and a hemagglutinin titer of 1:1024 as measured by the pattern test.[4] From the 7th amniotic passage on, when the hemagglutinin titer in the amniotic fluid exceeded 1:4096, a slight degree of hemagglutination became discernible in the allantoic fluids. Inoculation of amniotic fluid of the 9th passage into the allantoic sac of 8-day-old chick embryos resulted in some propagation of the agent in this cavity. After 5 days of incubation, the pooled allantoic fluids showed a hemagglutinin titer of 1:128.

The identity of the virus has been established in 3 ways. First, neutralization tests were performed in chick embryos with amniotic fluid of the 4th passage. Rabbit anti-mumps serum in dilution 1:10 neutralized 10,000 ID_{50} of the agent, whereas rabbit anti-influenza sera failed to do so. A human convalescent serum of high complement-fixing activity reacted to an extent comparable to that of the specific rabbit immune serum. Second, complement-fixation tests with the amniotic fluid of the 5th amniotic passage as antigen revealed strong reactions with known mumps convalescent sera but no, or lesser, reactions with sera taken from the corresponding patients during the acute stage of the disease. Finally, the same amniotic fluid (5th passage) was found to produce typical, although mild, parotitis in 2 out of 4 individuals who were judged susceptible to mumps by the results of complement-fixation tests prior to exposure.‡ All 4 cases developed antibodies to both the soluble and virus antigens. Thus, there was no doubt that the agent constituted a strain of mumps virus. This strain differed markedly from the one present in the laboratory at that time in the following respects: The new strain grew in the early passages only in the amniotic sac whereas the laboratory strain propagated equally well in the allantoic and amniotic cavities; also, the new strain gave rise to clinical mumps in exposed human subjects, while the laboratory strain produced only subclinical infections as demonstrable by rises in antibodies beginning on the 13th to 19th day after exposure.‡

Summary. A virus has been isolated from the spinal fluid of a patient with meningo-encephalitis without parotitis by inoculation of chick embryos by the amniotic route. This agent has been identified as mumps virus.

NOTE: Since this paper was written mumps virus has been recovered from the spinal fluid of another patient (T.P.) with meningo-encephalitis without parotitis. Positive red cell agglutination was obtained in second passage amniotic fluid. The finding of circulating complement-fixing antibodies against the soluble antigen (titer 1:16) but not against the virus antigen of the mumps virus established the diagnosis on the second day of illness.

[8] Levens, J. H., and Enders, J. F., *Science*, 1945, **102**, 117.

‡ These data, obtained in collaboration with Drs. Joseph Stokes, Jr., and Harriet Davis, will be published in greater detail separately.

16040 P

Effects of Radiotoxic Dosages of I^{131} upon Thyroid and Contiguous Tissues in Mice.*

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It seems remarkable that despite the extensive use of radioactive iodine in experimental and clinical studies no information has appeared in the literature concerning the radiation dosage from which injury may be expected in the thyroid gland. Such information seems implied in the use of I^{131} for the destructive irradiation of human thyroid carcinoma, but even here knowledge of a possible differential in radiation-sensitivity between normal and neoplastic thyroid tissue is greatly to be desired.

In an exploratory study, 36 inbred mice (A and C_{57} strains), 2 to 5 months old, and of both sexes, have been given "tracer" quantities of I^{131} in the form of NaI in neutral aqueous solution by subcutaneous injection. Mice were fed purina laboratory chow and kept at a constant temperature of $72° \pm 1°$F. Radiation dosages ranged from 100 to 1000 microcuries. Calculation of dosage was made from standardizations on a Geiger counter which, in a survey by the National Bureau of Standards in June, 1947, compared favorably in sensitivity to I^{131} with instruments at most other institutions. Mice were sacrificed 2, 3, 24, or 120 days after injection. Thyroids and surrounding tissues were serially sectioned.

Table I provides an approximate summary of the observations made.

A striking early effect of the localized radiation was a periglandular edema which separated the thyroid from surrounding structures and extended even into neighboring muscle. The edematous connective tissue was extensively infiltrated by lymphocytes, polymorphonuclear leucocytes, and some mast cells. Pycnosis of areas of tracheal epithelium was common. In animals killed on the second day following injection of 300 microcuries (20-23 millicuries I^{131} per kg) there was little effect beyond this. In the animal killed 2 days after receiving 53 mc per kg the thyroid appeared as an eosinophilic mass, amorphous in medullary parts, but with recognizable surviving follicles in the peripheral parts of the gland. Cells with still stainable nuclei either were pycnotic or else showed signs of activity (hypertrophy, exhaustion of colloid). In all instances surviving thyroid tissue was in the isthmus or the cranial apex of the gland where, presumably, self-radiation would have been minimal.

On the third day after injection thyroid destruction varied from minimal with lower dosages to complete with the 50 mc per kg dose. Parathyroid involvement paralleled these changes. Furthermore, dosages above 20 mc per kg produced pycnosis of nuclei of neurilemma and sheath cells in the recurrent laryngeal nerve, in those sections most intimately associated with the thyroid.

After a 24 day exposure to I^{131} radiation even the low dose (3-5 mc per kg) produced extensive destruction, and a medium dose (18-22 mc per kg) produced complete thyroidal destruction. At this time fibrosis had replaced the amorphous mixtures of epithelial debris and leucocytes with a somewhat edematous type of fibrous tissue. No normal parathyroid tissue remained.

At the 120 day interval the thyroid consisted of a shrunken fibrous band, the edema and leucocytic reaction having disappeared. In animals having received the low dose of I^{131} most glandular tissue had disappeared but a few surviving small follicles were imbedded in the fibrous tissue, and seemed to be secreting actively, and possibly serving as a reservoir for regeneration.

* This investigation has been aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

TABLE I.
Summary of Results.

Exposure to I^{131}, days	No. of mice	Dose, millicuries per kg	Approx. % thyroid destruction	Approx. % parathyroid destruction
2	2	20-23	0	0
2	1	53	90	75
3	6	3-5	10	0
3	4	17-18	75	25
3	4	30-35	80-90	75
3	3	50-55	100	95
24	8	3-5	25-50	10
24	4	18-22	100	50
120	4	3-4	90	100

Summary. Dosages of I^{131} from 3 to 50 millicuries per kilogram were given to young mice on a normal diet. Higher dosages produced complete thyroidal destruction within a few days. Lower dosages permitted survival of some thyroidal epithelium in the isthmus and cranial apex of the thyroid for as long as 120 days after injection, but resulted in loss of the parathyroids. Lesions were noted in the tracheal epithelium with all dosages, and in the recurrent laryngeal nerve with higher dosages.

16041 P

Thiouracil and Conversion of Carotene to Vitamin A Measured by Liver Storage in the Rat.*

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In a recent report, Canadell and Valdecasas[1] fed rats, which previously were on a vitamin A-free diet with thiouracil administered in the drinking water, 60 γ of carotene per week. The administered carotene was unable to relieve the ocular symptoms produced by a vitamin A deficiency. However, these symptoms were alleviated if small amounts of thyroid powder were administered with the carotene or vitamin A was fed to the thiouracil-treated animals. Their interpretation of this phenomenon was that it involved an inhibition of carotenase.

If this is true, then the feeding of carotene to vitamin A deficient-animals previously treated with thiouracil should produce little or no vitamin A in the liver. Therefore, this experiment was attempted, and a preliminary report of the results obtained is presented.

Twenty-six rats were placed on a vitamin A-low diet when they were 10 days old. When they reached a body weight of 43 g, they were divided into 2 groups. One group was continued on the same diet for 4 weeks while the second group received a similar diet to which 0.25% of thiouracil had been added. All rats were continued for an additional 2 weeks on identical diets except that they were made vitamin A-free by replacing the commercial casein with vitamin A-free test casein

* Aided by a grant from the Nutrition Foundation.

1 Canadell, J. M., and Voldecasas, F. G., *Experientia*, 1947, **3**, 35.

TABLE I.
Body Weight and Vitamin A Content of Young Rats Receiving a Vitamin A-free Diet With or Without Thiouracil at Periods of 36 Hours to 7 Days After Feeding 348 γ of β-carotene.

Carotene fed, γ	Rats administered diet containing 0.25% thiouracil			Control rats receiving no thiouracil		
	No. of rats	Body wt, g	Vit. A content per liver,* I.U.	No. of rats	Body wt, g	Vit. A content per liver,* I.U.
0	6	83	(4.5)†	6	156	(6.7)†
348	8	86.5	56.7 ± 24.4 (11.4-232)‡	6	143	56.2 ± 10.1 (17.9-91.5)‡

* Includes standard error of the mean calculated as follows:

$$\sqrt{\Sigma d^2/n} \;/\; \sqrt{n}$$

where "d" represents the deviation from the mean and "n" is the number of observations.
† Not considered as vitamin A since no fading blue color developed.
‡ Range of results.

(General Biochemicals, Inc.). Some of the animals in each group received a single oral dose of a solution of β-carotene in cottonseed oil containing 348 γ while the remaining rats, which served as controls, received cottonseed oil. The animals were sacrificed at 36 hours, 5 days or 7 days. Since the vitamin A levels in the different groups did not show any significant trend with time, the results obtained with animals sacrificed at 36 hours, 5 or 7 days have been averaged together. Vitamin A was extracted from the livers by the procedure employed by Mattson, Deuel and Mehl.[2] The determination of vitamin A was by the Carr-Price method using a Coleman Junior spectrophotometer. The results are summarized in Table I.

It would appear from these experiments that a thiouracil-treated rat can convert carotene to vitamin A and store this product in the liver when fed 348 γ of carotene. However no results on the animals' ability to utilize this stored vitamin A are presented. Drill and Truant[3] in a recent paper, using thyroidectomized animals, could not prevent or alleviate ocular symptoms by injecting 10 γ of carotene per day. However, Remington *et al.*[4] found that an oral dose of 0.6 γ of carotene per day was able to bring about a cure of the eye symptoms of thyroidectomized rats within 7-9 days. No difference between the effectiveness of vitamin A and carotene was noted in these experiments.

Experiments are now under way to demonstrate the lowest level of carotene and vitamin A which must be fed to a thiouracil-treated animal to alleviate the eye symptoms from vitamin A deficiency.

[2] Mattson, F. H., Mehl, J. W., and Deuel, H. J., Jr., *Arch. Biochem.*, 1947, **15**, 65.

[3] Drill, V. A., and Truant, A. P., *Endocrinology*, 1947, **40**, 259.

[4] Remington, R. E., Harris, P. L., and Smith, C. L., *J. Nutrition*, 1942, **24**, 597.

16042

Antipyridoxine Activity of Methoxypyridoxine in the Chick.

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Desoxypyridoxine (2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine), an analogue of pyridoxine, has been shown to be a strong inhibitor of pyridoxine in chicks[1] and to exhibit a similar but less potent effect in rats.[2] Further studies on additional analogues of this vitamin have led to the discovery that 2-methyl-3-hydroxy-4-methoxymethyl-5-hydroxymethylpyridine (methoxypyridoxine) also has pronounced antipyridoxine activity in chicks. In rats, however, this methoxypyridoxine has some vitamin activity,[3] presumably due to the ability of this species to convert part of the compound to pyridoxine.[4]

The procedure employed in studies on the effect of methoxypyridoxine in chicks was similar to that described previously.[1] To begin an assay, eight-day-old chicks weighing approximately 60 g after maintenance for 5 days on a purified diet (Table I) deficient in pyridoxine were distributed into groups balanced in regard to body weight. The chicks were dosed orally with the test substances on the first, third, fifth and seventh days of the assay period. Body weights were recorded on these days as well as on the ninth (last) day of the test. The pyridoxine-deficient diet was fed to all groups during the assay period.

The curve of response (weight gain *vs.* log dose) was established for each assay by using 3 or more of the groups of chicks on dosages in the range from 5 to 50 micrograms pyridoxine per dose. The growth response of each group receiving a combination (premixed solution) of methoxypyridoxine and pyridoxine was compared with the curve of response for pyridoxine to determine the net pyridoxine activity of the combination. The inhibition ratio between analogue and vitamin was then calculated from the amount of methoxypyridoxine administered and the apparent loss in pyridoxine activity.

TABLE I.
Pyridoxine-deficient Diet for Chicks.

	g
Dextrose (cerelose)	51.5
Casein (vitamin free)	25.0
Salts IV[5]	5.0
Cellulose (ruffex)	5.0
Calcium gluconate	2.5
Glycine	2.0
Liver extract L*	2.0
KH_2PO_4	1.0
Wheat Germ Oil	4.5
400 D fish liver oil	0.5
Arginine	0.5
Cystine	0.2
Choline	0.2
Inositol	0.1
p-Aminobenzoic acid	0.03
Niacin	0.01
Calcium pantothenate	0.004
Riboflavin	0.002
Thiamine	0.002
Menadione	0.0004
Biotin	0.00004
Total	100.05

* Wilson & Co., Inc., Chicago, Ill.

A single dose of 200 μg of methoxypyridoxine killed all the chicks in the group within 48 hours after administration (Table II). Even quantities as low as 15 μg per chick were fatal in the absence of pyridoxine. This toxic effect was prevented by simultaneous administration of an equal amount of pyridoxine, thus indicating that methoxypyridoxine had antipyridoxine activity. The bioassays (Table II) showed that approximately 4 parts by weight of methoxypyridoxine hy-

1 Ott, W. H., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 125.

2 Emerson, G. A., unpublished data.

3 Unna, K., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 122.

4 Porter, C. C., Clark, I., and Silber, R. H., *J. Biol. Chem.*, 1947, **167**, 573.

5 Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

TABLE II.
Antipyridoxine Activity of Methoxypyridoxine in 8-day Curative Assays with Chicks Receiving a Pyridoxine-deficient Diet.

Exp.	Oral Supplement* Pyridoxine hydrochloride, μg	Oral Supplement* Methoxypyridoxine hydrochloride, μg	Survival of chicks, alive/total	Wt gain per chick, g	Loss in pyridoxine activity, μg	Ratio of inhibition, analogue : vitamin
1	0	200	0/6	†		
	0	0	4/7	11.4		
	20	0	7/7	50.9		
2	0	15	0/7	‡		
	0	0	4/7	10.0		
	7.5	0	2/7	34.7		
	15	0	7/7	38.8		
	50	0	7/7	46.7		
	15	15	7/7	43.3	0	—
	15	30	5/7	33.5	8	4:1
	15	45	5/7	28.1	11	4:1
3	20	40	1/7	—	20	2:1
	20	60	5/7	20.8	16	4:1
	20	80	5/7	22.9	15	5:1
	0	0	6/7	14.5		
	5	0	14/14	24.9		
	10	0	13/14	31.6		
	20	0	6/7	37.1		
	40	0	7/7	56.2		
4	60	60	7/7	36.6	24	2.5:1
	60	120	3/7	26.0	42	3:1
	60	240	6/7	17.5	50	5:1
	60	360	3/7	8.7	55	6.5:1
	0	0	3/7	4.0		
	10	0	14/14	17.7		
	40	0	14/14	38.6		

* Amount given per chick on each of 4 successive alternate days.
† All dead after 1st dose.
‡ 2 dead after 1st dose, 3 dead after 2nd dose, 2 dead after 3rd dose.

drochloride counteracted the biological activity of one part by weight of pyridoxine hydrochloride. In the chick, therefore, methoxypyridoxine is approximately as powerful an inhibitor of pyridoxine as is desoxypyridoxine.[1]

The inhibition ratio of 2:1 reported for desoxypyridoxine[1] is not significantly different from the inhibition ratio of 4:1 estimated above for the methoxy analogue of pyridoxine. Nevertheless, there seems to be a difference in the antivitamin action of the two analogues. Although both compounds appear to compete with pyridoxine, it has been observed on the basis of limited tests that the effects of lethal doses of desoxypyridoxine were relatively easily counteracted by administration of pyridoxine even after a considerable length of time. On the other hand, when a lethal dose of methoxypyridoxine had been given, subsequent administration of pyridoxine was generally ineffective in preventing the death of the chicks. These and other observations on comparative antipyridoxine activity in the chick are being investigated further.

Summary. 2-Methyl-3-hydroxy-4-methoxymethyl-5-hydroxymethylpyridine (methoxypyridoxine) has been shown to have the same order of antipyridoxine activity as desoxypyridoxine in chicks. In these experiments, approximately four moles of methoxypyridoxine counteracted the vitamin activity of one mole of pyridoxine when suboptimal or optimal amounts of the vitamin were given to pyridoxine-deficient chicks.

16043

Estimation of Alterations of Serum Gamma Globulin by a Turbidimetric Technique.

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Increased attention has been devoted in recent years to the elevation of serum globulin encountered in patients with diseases of the liver. The wide use of the cephalin flocculation and the thymol turbidity reactions for the diagnosis of liver disease has been partially responsible for this renewed interest. These reactions have been shown to depend mainly on small changes in the globulin fraction of the serum.[1,2] However, other factors are involved and the exact aberration of the serum that is measured has not been completely identified. In the case of the thymol turbidity test, for example, the concentration of the serum lipids has a definite effect on the intensity of the reaction.[2] It would seem important, therefore, to determine specifically and directly slight elevations of globulin that occur in acute liver disease.

The usual methods of globulin estimation which depend on nitrogen determinations following salting out procedures are subject to considerable error. The technique is time consuming and minor changes in the globulin level may not be apparent. Changes in the amount of serum globulin can best be detected at the present time by means of electrophoretic patterns. Such a technique, however, is not practical for routine application.

Dilution of serum with solutions of low ionic strength decreases the solubility of the proteins and under certain conditions the more insoluble globulins will precipitate. This technique is utilized in the thymol turbidity test[3] and in the buffer dilution test of Wolff.[4] Both of these reactions are positive in acute liver disease and appear to depend partly on increases in the gamma globulin fraction of the serum. In certain conditions, such as cirrhosis of the liver, large increases in gamma globulin may be present in the serum in the absence of increased values for these two tests. The intensity of these reactions, therefore, is not always proportional to the increase in amount of gamma globulin in the serum.

The present paper describes a turbidimetric technique which provides an index of the degree of elevation of the gamma globulin fraction of the serum of patients irrespective of their disease. Dilution of serum with solutions of the salts of heavy metals was found to precipitate various protein fractions according to the concentration of metal used. Fig. 1 illustrates the curve of protein precipitation from normal serum at various concentrations of $CuSO_4$. Electrophoretic analyses showed that at the lower concentrations the precipitate consisted almost entirely of gamma globulin. By means of this curve it was possible to estimate a concentration of $CuSO_4$ that caused minimal precipitation of protein from normal serum. When hepatitis serum showing slight elevation of the gamma globulin fraction was diluted with $CuSO_4$ solution of this concentration, precipitation occurred, and the amount of precipitate was proportional to the increase in gamma globulin. The precipitated globulin could be accurately determined by turbidity measurements because the protein precipitated as a diffuse finely particulate suspension.

Zn, Hg, Pb, Cd, and Ur salts were found to produce an effect similar to that of $CuSO_4$.

[1] Moore, D. B., Pierson, P. S., Hanger, F. M., and Moore, D. H., *J. Clin. Invest.*, 1945, **24**, 292.

[2] Kunkel, H. G., and Hoagland, C. L., *J. Clin. Invest.*, 1947, **26**, 1060.

[3] MacLagan, N. F., *Brit. J. Exp. Path.*, 1944, **25**, 234.

[4] Wolff, E. K., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1939, **32**, 707.

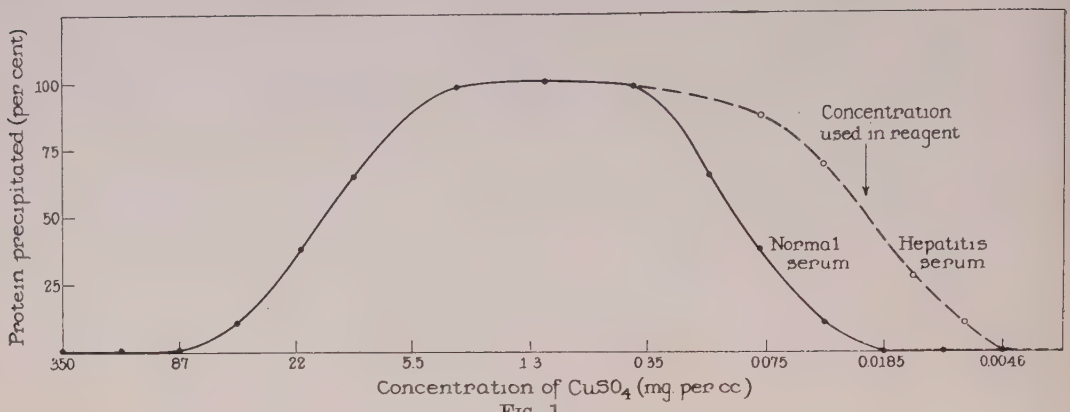

FIG. 1.

Per cent of the total protein of normal and hepatitis serum precipitated following a 60-fold dilution with various concentrations of $CuSO_4 \cdot 5H_2O$ in distilled water.

Curves illustrating the effects of various concentrations on the precipitation of protein from serum closely resembled the curve for $CuSO_4$ in Fig. 1. When buffered solutions were used, $ZnSO_4$ was found to have certain advantages over $CuSO_4$.

The effect of the solutions of heavy metals in precipitating serum proteins was altered by small changes in pH and ionic strength. The results in Table I illustrate the comparative effect of the $CuSO_4$ solution usually used as reagent on normal and hepatitis serum under various conditions. The best differentiation between the two types of serum occurred at pH 6.5-7.5 in the presence of as low an ionic strength as it was practical to use.

Various concentrations of $CuSO_4$ reagent could be used for estimating changes in the globulin fraction. The concentration of 23 mg of $CuSO_4 \cdot 5H_2O$ per liter which causes minimal precipitation with normal serum was found to be the most useful. A zinc sulfate solution containing 24 mg of $ZnSO_4 \cdot 7H_2O$ per liter gives similar results, and has the advantage that it can be permanently buffered with barbiturate, whereas copper sulfate solution with barbiturate forms a precipitate. For routine use the solution eventually employed contained, per liter, 24 mg of $ZnSO_4 \cdot 7H_2O$, 280 mg of barbituric acid, and 210 mg of sodium barbiturate; the pH was 7.5.

Procedure. Measure one volume of serum (0.05 ml) into 60 volumes of either the $CuSO_4$ or the buffered $ZnSO_4$ reagent (3 ml). Allow to stand for 30 minutes, shake, and then read the turbidity in the spectrophotometer at 650 mμ.

The turbidity produced was translated into units by applying a standard curve similar to the one used for the thymol turbidity test.[5]* All values reported in this paper were determined by means of a Coleman Jr. spectrophotometer at 650 mμ. A colorimeter can also be used. In the absence of these instruments, a rapid estimate of the globulin elevation can be made by direct visual observation of the turbidity produced. The time necessary for flocculation to occur was a simple index of the globulin elevation. Some of the sera showing very high globulin levels demonstrated flocculation in a few minutes. The buffered $ZnSO_4$ reagent produced flocculation somewhat more rapidly than did

[5] Shank, R. E., and Hoagland, C. L., *J. Biol. Chem.*, 1946, **162**, 133.

* 3 cc of a $BaCl_2$ solution (containing 1.15 g $BaCl_2 \cdot 2H_2O$ per 100 cc) is made to 100 cc in a volumetric flask with 0.2*N* H_2SO_4. This $BaSO_4$ suspension gives a turbidity equivalent to 20 units. By assigning the value 20 units to the optical density reading obtained in a colorimeter or a spectrophotometer at 650 μ with the $BaSO_4$ suspension, a standard curve can be constructed by drawing a line through the point obtained in the above manner and the 0 point on ordinary graph paper. The type of cuvette is not important as long as the same type is used for constructing the standard curve as for routine readings.

TABLE I.
Turbidity Produced by Adding 3 cc of a 2.3 mg % Solution of $CuSO_4$ to 0.05 cc of Serum Under Different Conditions of pH and Ionic Strength. Turbidities Are Expressed in Units Described in Text.

pH	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.2	7.5	8.0	8.5	Ionic strength
Turbidity														
Normal serum	0	0	0	2	4	13	19	14	9	6	2	0	0	.01
Hepatitis serum	0	2	3	4	6	19	31	31	31	24	19	0	0	
Normal serum	0	0	0	0	0	0	4	2	0	0	0	0	0	0.1
Hepatitis serum	0	0	0	0	0	4	9	6	5	3	0	0	0	

the $CuSO_4$ reagent. Normal serum did not flocculate for at least 12 hours with the $ZnSO_4$ reagent while serum from patients with very slight elevation of the gamma globulin fraction usually flocculated within 4 hours.

The protein precipitated in the reaction dissolved readily on dialysis against sodium cyanide and the latter could be removed by further dialysis against barbital buffer at pH 7.8. Electrophoretic analysis of the protein treated in this manner revealed that the chief component precipitating in the reaction was a gamma globulin plus small amounts of other fractions (Fig. 2). This was true of the precipitate from 4 different sera that were studied. It appeared as if small amounts of other protein fractions, which normally would not precipitate at such a concentration of metallic salt, were carried down with the gamma globulin of these abnormal sera. Numerous observations of electrophoretic patterns were carried out on whole sera from patients showing different values for the copper and zinc turbidity reactions. In every case the increase in the gamma globulin component correlated well with the intensity of the reaction. Fig. 3 illustrates the close relationship between the turbidity as measured by dilution of serum with the zinc reagent and the gamma globulin concentration as calculated from electrophoretic patterns of various pathological sera showing approximately normal albumin levels.

Certain sera with elevated lipid levels showed a marked increase in the beta globulin fraction. This increase was not reflected in the copper turbidity reaction. It is well known that such beta globulin peaks will often disappear almost completely by extraction of the lipids from the serum[6] and do not represent a true picture of the protein migrating in this fraction.

Addition of albumin to a positively reacting serum decreases the amount of globulin precipitated. Fig 4 demonstrates the *in vitro* change in the turbidity produced with the copper reagent upon the addition of increas-

[6] Longsworth, L. G., and MacInnes, D. A., *J. Exp. Med.*, 1940, **71**, 77.

FIG. 2.
Ascending and descending electrophoretic patterns of the protein precipitated in the copper turbidity reaction. The major peak has the mobility of gamma globulin.

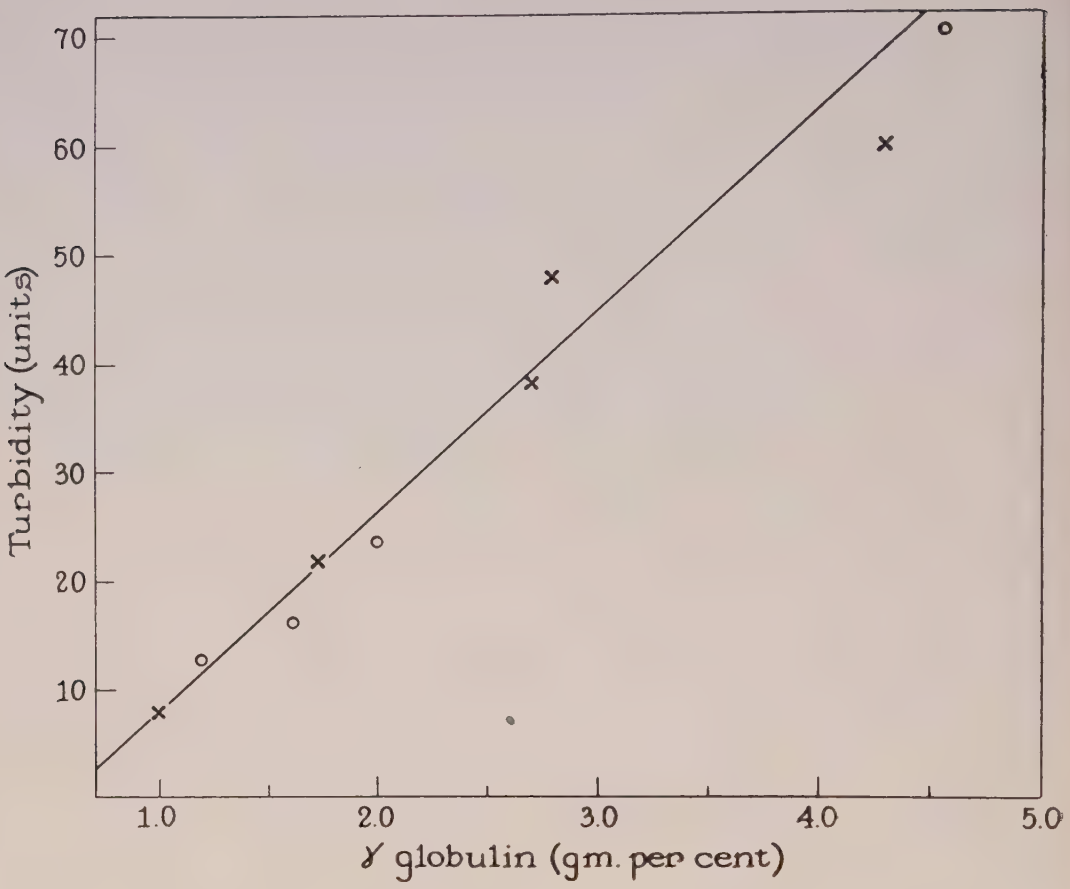

FIG. 3.
The correlation between the turbidity as measured in the zinc turbidity reaction and the gamma globulin concentration as calculated from electrophoretic patterns of various pathological sera. X = liver disease; O = other conditions.

ing amounts of human serum albumin. The intravenous administration of large amounts of concentrated human albumin to patients with liver disease demonstrated that a slight fall in the intensity of the reaction occurred and that it was similar to the fall expected from the *in vitro* experiments. The fact that an albumin deficit without hyperglobulinemia will not cause the copper turbidity reaction to become positive is clear, however, from observations on patients with nephrosis. The copper turbidity reaction was found normal despite the albumin deficit found in this disease.

Approximately 1000 determinations with the copper and zinc turbidity reactions have now been carried out on various sera. The normal range was found to lie between 2 and 8 units. Certain sera with globulin levels above 6 g % have shown values as high as 80 units. In these sera more than half of the protein was precipitated. Fig. 5 illustrates the close relationship between the turbidity in units and the globulin level of the serum. The reaction is not specific for liver disease but depends on the degree of elevation of gamma globulin. Serum from patients with multiple myeloma, for example, showed turbidity in proportion to the increase in gamma globulin. Forty-one patients with cirrhosis of the liver were tested and all were found to demonstrate a positive reaction. The test was of particular value when applied serially throughout the course of an acute illness. Fig. 6 shows the markedly delayed elevation in gamma globulin as determined by the zinc turbidity test

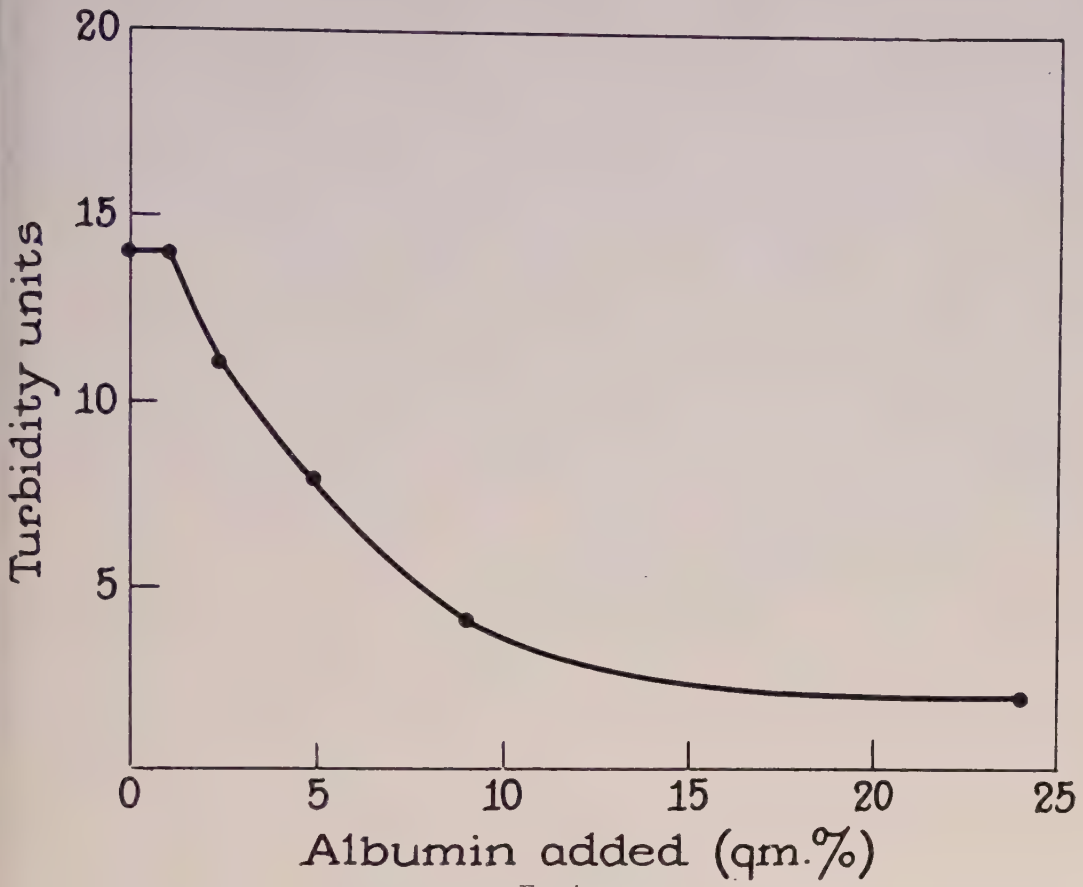

FIG. 4.
The effect of adding increasing amounts of concentrated human albumin to hepatitis serum on the intensity of the copper turbidity reaction.

during the course of a recurrence of acute infectious hepatitis. Similar changes with a delayed rise and prolonged elevation of the gamma globulin level have also been observed following an uncomplicated attack of acute infectious hepatitis.

Discussion. The Takata-Ara reaction and its many modifications, which have long been utilized for the diagnosis of liver disease, depend on the precipitation of protein from serum with a high globulin level. The mechanism of these reactions has always been obscure, but one of the substances added to serum in performing the tests is the metallic salt, $HgCl_2$. The study of the effect of various concentrations of heavy metals on the precipitation of protein from serum has furnished an understanding of this reaction. Curves similar to the ones illustrated in Fig. 1 for $CuSO_4$ may be constructed for various mercury salts and concentrations which will precipitate globulins from abnormal sera may be obtained. The Takata-Ara reaction does not employ the dilution technique and is only positive when marked elevation of the globulin level occurs.

The concentrations of zinc and copper sulfates used in the reagents described above were useful for detecting elevations in gamma globulin in liver disease. However, other concentrations may also be used for different purposes. For example, a slightly higher concentration will precipitate globulin from serum with a normal or an abnormally low globulin level. The procedure of determining protein aberrations by the turbidity developed in dilute, metallic salt solutions is so simple in use and consistent in results that it may find

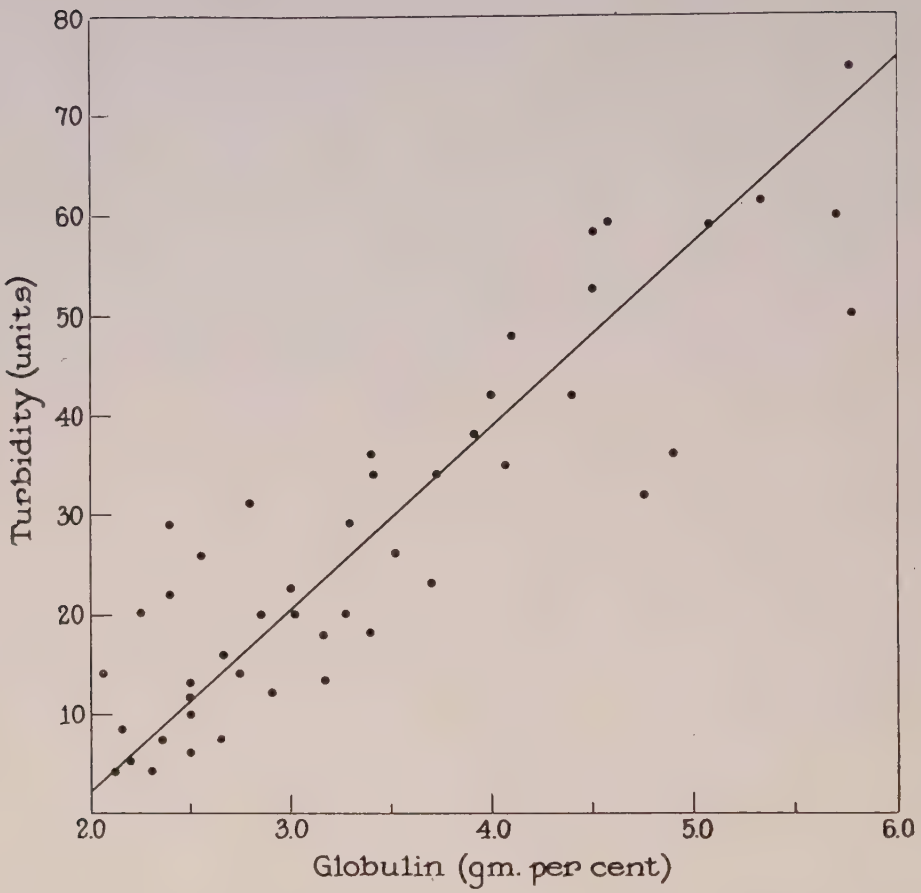

FIG. 5.

The correlation between the turbidity as measured in the zinc turbidity test and the total globulin level as determined by the Howe fractionation in various pathological sera.

application for a wide variety of purposes.

Measurable increases in the total globulin of serum in liver disease and in other conditions are almost always due to elevation of the gamma globulin fraction.[2,7] The beta globulins may show significant changes electrophoretically but these depend mainly on the lipid level of the serum and do not represent true protein alterations. As a result, any technique which measures increases in gamma globulin in pathological sera is really a measure of the elevation in total globulin.

Significant curves of the globulin changes during the course of acute infectious hepatitis were not obtained until the copper and zinc turbidity techniques were adopted. The delayed rise and prolonged elevation during the course of this disease is of considerable interest. It suggests the possibility that the globulin elevation may reflect the production of antibodies rather than a disturbance in liver function. The albumin level of the serum was often unaltered during the period of hyperglobulinemia. Serial determinations of the thymol turbidity test closely paralleled those of the globulins during the convalescent phase of infectious hepatitis, but marked differences were readily apparent in cases of cirrhosis of the liver. In this condition the thymol turbidity reaction was of considerably less value and showed little relation to the globu-

[7] Gray, S. J., and Guzman Barron, E. S., *J. Clin. Invest.*, 1943, **22**, 191.

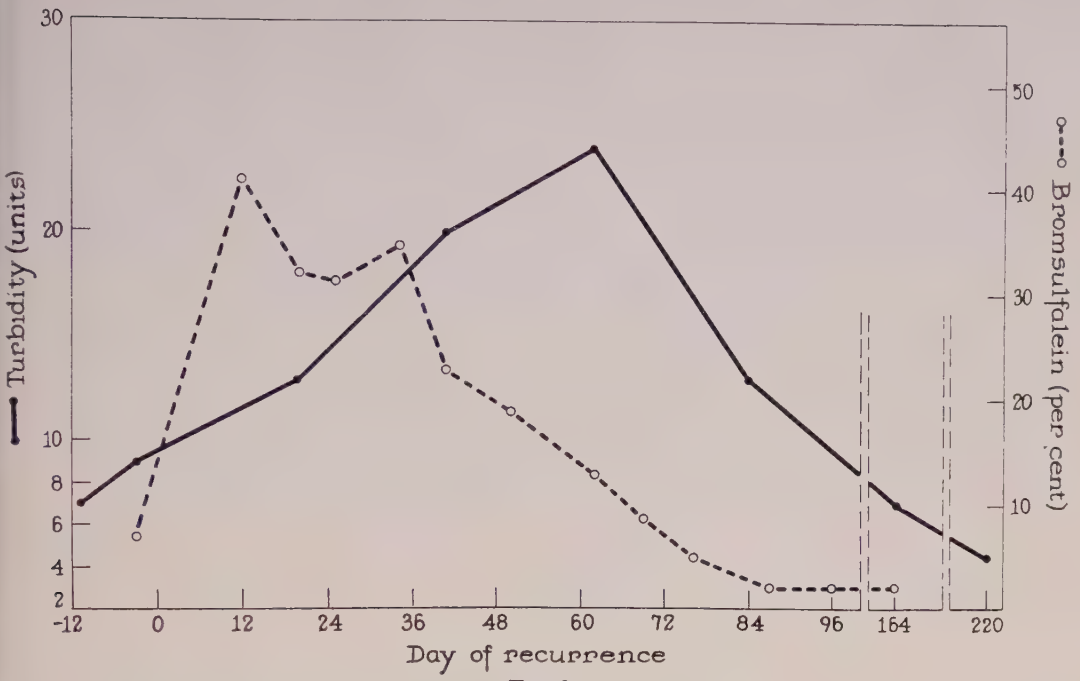

FIG. 6.

The delayed rise and prolonged elevation of the gamma globulin level in the serum following a recurrence of infectious hepatitis as determined by the zinc turbidity test. The curve for bromsulfalein retention is added for purposes of comparison.

lin aberration.[8]

In chronic infectious hepatitis alterations in the globulin components of the serum may be the only indication of persisting liver disease. In previous studies[8,9] it was demonstrated that the thymol turbidity test was positive more often than any other single liver function test that could be applied to patients with persistent vague complaints more than 6 months after an attack of acute infectious hepatitis. Electrophoretic patterns showed that values for the thymol test paralleled the changes in the gamma globulin fraction closely in these patients. All patients with abnormalities in the thymol test also showed abnormalities in the copper and zinc turbidity reactions. In addition, the latter frequently remained positive after the thymol test had become negative. The zinc turbidity test, although not specific for liver disease, was found to be the most sensitive method of detecting a lingering hepatitis. Five patients have been observed who developed cirrhosis of the liver following infectious hepatitis. A characteristic feature of this group was marked elevation of the gamma globulin fraction of the serum electrophoretically. In two of these patients the gamma globulin reached values above 6 g %. These aberrations were easily detected by the addition of the metal reagents to serum. The use of this test provided a rapid method of screening patients with persistent symptoms following infectious hepatitis in searching for the more severe complications of the disease.

The main advantage in the use of a test of this type instead of the cephalin flocculation or the thymol turbidity reaction is that a single known alteration in the serum is measured. This alteration is an elevation in the gamma globulin fraction. The other reactions depend partly on this alteration but also on other factors less well understood, such as the lipid level of the serum.

Another application of the zinc turbidity test has been in the study of the gamma globu-

[8] Kunkel, H. G., and Hoagland, C. L., *Am. J. Med.*, in press.

[9] Kunkel, H. G., and Hoagland, C. L., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 258.

lin changes associated with the development of antibodies following scarlet fever in patients with normal convalescence and patients developing rheumatic fever. Increased turbidity was found to closely parallel the rise in antistreptolysin and antistreptokinase titers. These results will be reported in a separate communication.

Summary. 1. When serum with abnormally high gamma globulin concentration is diluted with a solution containing a certain small amount of copper or zinc sulfate, a turbid precipitate forms and the optical density of the suspension is proportional to the concentration of gamma globulin.

2. Such an estimation of increase in gamma globulin really measures the total globulin elevation in pathological sera because hyperglobulinemia is almost always due to an alteration in the gamma globulin fraction of the serum.

3. The test has proved useful for determining alterations in gamma globulin during the course of an acute illness such as infectious hepatitis.

4. It was found to be of particular value in detecting persistent liver disease following infectious hepatitis. In a group of 41 patients with cirrhosis of the liver the reaction was positive in every case.

16044 P

Black Pigmentation in Feathers of Buff Orpington Chicks Caused by Vitamin D Deficiency.*

ANNABELLE DECKER AND JAMES MCGINNIS. (Introduced by A. R. Kemmerer.)

From the Department of Poultry Husbandry, State College of Washington, Pullman, Wash.

Abnormal amounts of black pigment in feathers of New Hampshire chicks fed vitamin D deficient diets were observed by Glazener, Mattingly, and Briggs.[1] Domm[2] reported that feeding desiccated thyroid caused abnormal black coloring in Brown Leghorn males, females, and capons. Juhn and Barnes,[3] working with capons of the same breed, produced similar results. Both the New Hampshires and the Brown Leghorns normally have some black pigment in the feathers. Buff Orpingtons, on the other hand, do not normally have black pigment in the feathers. It was of interest, therefore, to determine whether a deficiency of vitamin D would cause the black-pigmented feathers in this breed.

Experimental Methods. The percentage composition of the vitamin D deficient basal diet fed in this experiment is as follows: ground yellow corn 30.0, ground wheat 25.5, ground barley 10.0, dehydrated alfalfa 5.0, B-Y riboflavin concentrate (250 μg riboflavin/g) 0.5, ground limestone 2.0, dicalcium phosphate 1.5, soybean oil meal (expeller) 20.5, fish meal 4.0, salt (iodized) 0.5, and soybean oil 0.5. Manganese was added at a level of 75 p.p.m.

Chicks were examined and weighed at weekly intervals. At 6 weeks the feather color of each chick was given a numerical score, ranging from 0 to 5 depending upon the intensity of black pigment in the back feathers. A score of 0 indicated no black pigment whereas a score of 5 indicated very markedly blackened feathers. Since some of the chicks were extremely slow feathering, it was not possible to score all of them.

At the end of the 6-week period, a few chicks from each vitamin D deficient diet

* Published as Scientific Paper No. 726, College of Agriculture and Agricultural Experiment Stations, Institute of Agricultural Sciences, State College of Washington, Pullman, Wash.

1 Glazener, E. W., Mattingly, J. P., and Briggs, G. M., *Poultry Sci.*, 1946, **25**, 85.

2 Domm, L. V., *Anat. Rec.*, 1929, **44**, 227.

3 Juhn, M., and Barnes, B. O., *Am. J. Physiol.*, 1931, **98**, 463.

TABLE I.
Effect of Vitamin D Deficiency on Feather Pigmentation in Buff Orpington Chicks.

Supplement to basal diet	Avg wt at 6 wk g		No. of chicks scored	Feather color: % showing black		Feather color: Color score	
None	312	304*	12	100	100*	3.1	2.9*
	296		13	100		2.6	
Vit. D†	482	470	13	0	0	0	0
	458		11	0		0	
Iodinated casein‡	318	309	11	100	100	2.0	2.7
	295		12	100		3.3	
Iodinated casein + vit. D	422	471	11	0	0	0	0
	520		9	0		0	

* Average of duplicate groups.
† 100 A.O.A.C. units/100 g diet.
‡ 15.0 g/100 lb diet.

were placed on a ration containing adequate vitamin D, and were observed from day to day for several weeks.

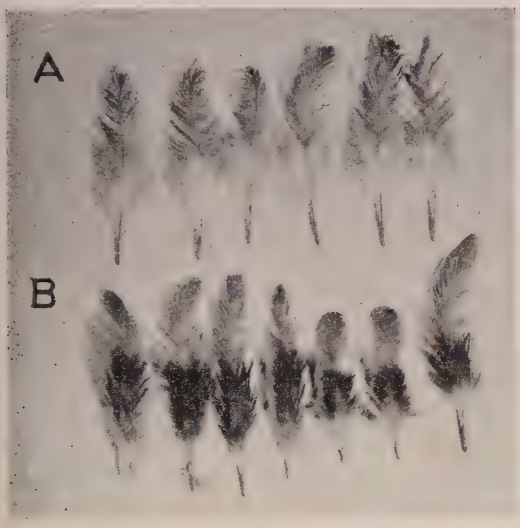

FIG. 1.
Black banded feathers of Buff Orpington chicks fed vitamin D-deficient diets. (A) Normal feathers taken from birds receiving adequate amounts of vitamin D. (B) Abnormal feathers from birds receiving vitamin D-deficient diets. Note banding effect caused by growth of new feathers of normal color after vitamin D supplementation.

Results. The results are presented in Table I and Fig. 1. At 4 weeks of age most of the chicks fed on the vitamin D deficient diets showed varying amounts of gray to black color. At 6 weeks of age intense black was noticeable in the feathers of the humeral tract of the vitamin D deficient birds. The undercolor of the back, breast, and leg feathers as well as color at the base of the flight feathers and wing coverts, was black. All of the chicks fed the vitamin D deficient diets grew some feathers with black pigment. The degree and distribution of black pigmentation, however, varied considerably. Without exception, all of the chicks receiving the diets supplemented with vitamin D or vitamin D plus iodinated casein had normal buff colored feathers, free of black pigment. Under the conditions of this experiment, thyroactive iodinated casein did not increase the deposition of black pigment in chicks fed a vitamin D deficient diet or a diet supplemented with this vitamin.

At the end of the experiment, several chicks from each lot were placed on a chick ration containing vitamin D. The immature black feathers of chicks fed the vitamin D deficient diets during the experimental period grew

with a normal buff color at the base following the change in diets. This growth of normal colored feathers was noted at about five days after the diets were changed. Fig. 1 shows the banding effect produced in the feathers.

Summary. In an experiment with Buff Orpington chicks, a breed normally having no black pigment in the feathers, it was found that a deficiency of vitamin D caused a widespread deposition of black pigment in the feathers. This abnormal blackening was prevented by supplementing the diet with vitamin D.

The feeding of thyroactive iodinated casein failed to increase the deposition of black pigment in chicks fed either a vitamin D deficient or vitamin D supplemental diet.

The iodinated casein was supplied by Cerophyl Laboratories, Inc., Kansas City, Mo.

16045

Response of Spontaneous Lymphoid Leukemias in Mice to Injection of Adrenal Cortical Extracts.*†

L. W. LAW AND ROBERT SPEIRS. (Introduced by C. C. Little.)

From the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and the Department of Zoology, University of Wisconsin, Madison.

Striking alterations in blood elements have been observed to result from injections of pituitary adrenotropic hormone in various species of animals.[1,2] The blood picture observed within a few hours after injection is as follows: decrease in leukocyte count, an absolute lymphopenia and a corresponding absolute polymorphonuclear leukocytosis. Statistically significant decreases in the weights of lymphoid tissues, excepting the spleen, following injections of adrenotropic hormone, indicate a profound influence on normal maintenance of lymphoid tissue by this hormone.[3] Histological studies indicate specific degenerative changes of normal lymphocytes in all lymphatic structures with repair and recovery after definite time intervals.[4] These changes are mediated through the adrenal cortex and similar alterations, although at possibly different time intervals, occur following injections of adrenal cortical extracts.[2]

In view of these profound effects on normal lymphocytic tissues, we have undertaken a study of the effects of adrenal cortical extract on the immature elements in spontaneous lymphoid leukemias in mice. Earlier studies have indicated some relationship between the adrenals and the growth of transmitted animal leukemias. Sturm and Murphy[5] showed that adrenalectomy reduced the natural resistance of rats to a transplantable lymphoid leukemia. The rate of growth of the leukemia was also apparently increased following adrenalectomy. It has been also[6] shown that desoxycorticosterone acetate and two adrenal cortical extracts decreased the susceptibility of rats to a transplanted leukemia. Complete disappear-

* Work supported in part under a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.

† Technical assistance of Lester E. Bunker, Jr., is gratefully acknowledged.

1 Dougherty, T. F., and White, A., *Science*, 1943, **98**, 367.

2 Dougherty, T. F., and White, A., *Endocrinology*, 1944, **35**, 1.

3 Dougherty, T. F., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 132.

4 Dougherty, T. F., and White, A., *Am. J. Anat.*, 1945, **77**, 81.

5 Sturm, E., and Murphy, J. B., *Cancer Research*, 1944, **4**, 384.

6 Murphy, J. B., and Sturm, E., *Science*, 1944, **99**, 303.

ance of a transplantable lymphoid tumor in mice followed by recurrence but definite retardation in growth after administration of 11-dehydro-17 hydroxycorticosterone (Compound E) has been reported.[7]

In the C58 strain of mice approximately 90% of mice of both sexes develop leukemia, the majority of which are lymphoid in origin. Early symptoms of leukemia can be detected within the strain by periodic palpation of lymph nodes. Usually a single node is initially involved[8] followed by a progressive systemic course of the disease. An incidence of approximately 80% leukemia, involving a significantly greater number of females than males, has been observed in the inbred RIL strain. The majority of these leukemias involve initially and principally the thymus. The first symptom observed is usually that of dyspnea and the animals are at this time in the terminal stage of the disease.

The leukocyte count is definitely elevated in leukemias of both strains of mice, although not severely, and immature lymphoid forms have been found in the peripheral blood of all leukemias observed within these strains. There occurs a progressive leukocytosis and a definite increase in circulating immature forms as the disease runs its course.

In the following experiment we are reporting 13 cases of spontaneous lymphoid leukemia arising in the inbred leukemic strains of mice, C58 and RIL (including 2 hybrids between these strains) which were injected with adrenal cortical extracts. Most of these leukemias were in the terminal stages of the disease (from 5 to 16 days following discovery of symptoms) when administration of adrenal cortical extract was begun. Mice of the C58 strain had moderate to severe lymphadenopathy of the axillary, inguinal and cervical lymph-nodes, moderate to severe splenomegaly and numerous immature leukocytes in the peripheral circulation. Mice of the RIL strain showed dyspnea and thoracic enlargement and in some cases had subcutaneous lymph-node and splenic involvement. In 5 of the 13 cases of spontaneous leukemia, biopsy tissue (axillary or inguinal lymph node) was taken prior to treatment, part of which was saved for histological section and the other part inoculated into mice of the strain of origin of the leukemia. This was done to confirm further the diagnosis of leukemia.

Leukemic mice were inoculated initially either intraperitoneally or subcutaneously with from 0.1 to 0.5 cc of adrenal cortex extract (aqueous) or lipoadrenal cortex.‡ Since the lipoadrenal cortical extract gave the most favorable effects, subsequent inoculations were continued with this at usually 0.1 cc inoculation every 24 hours. Several animals received this dose every 12 hours for a considerable period of time and optimum response as determined by blood analyses was obtained. Blood analyses were made at 0, 3, 6, 9, 24, 72, and 168 hours and at various times thereafter throughout the life of the animal.

The blood picture obtained is characterized by: (a) decrease in total white blood-cell count, (b) decrease in absolute number of lymphocytes with a corresponding polymorphonuclear leukocytosis and decrease in absolute number of immature forms (lymphocytes and lymphoblasts). Following initial administration of adrenal cortical extract, the maximum blood alterations were noted at 6 hours. After continued hormone administration these effects were enhanced so that in the majority of mice given daily injections for 168 hours or longer there resulted a severe lymphopenia and decrease in absolute number of circulating immature blood cells. Two cases, however, both in the C58 strain proved refractory after 72 hours and showed a progressive increase in circulating leukocytes and immature forms. Recovery to a leukemic blood picture tended to occur after 9 hours following injection of adrenal cortical extract. However, after continuous daily injections over a relatively long period of time an effect of the extract has been observed in animals 24 hours after injection

[7] Heilman, F. R., and Kendall, E. C., *Endocrinology*, 1944, **34**, 416.

[8] Law, L. W., *Proc. Nat. Acad. Sci.*, 1947, **33**, 204.

‡ Obtained from the Upjohn Co., Kalamazoo, Mich.

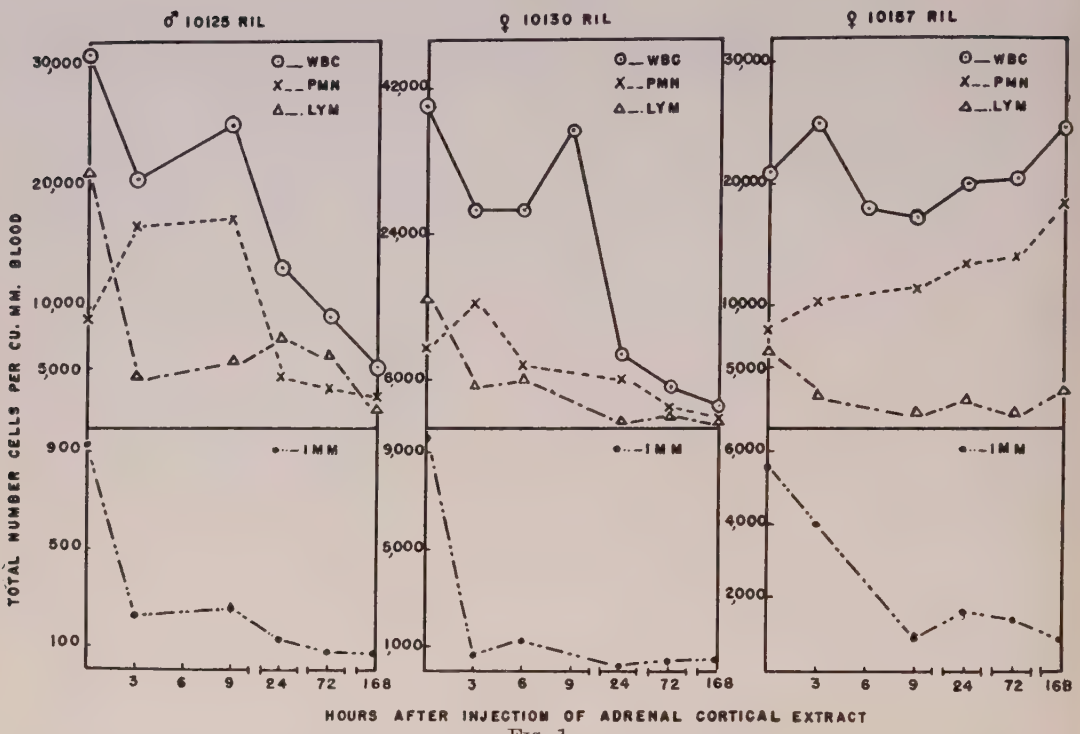

FIG. 1.

Effect of injections of adrenal cortical extract on the white blood cell counts of three mice of the RIL strain with lymphoid leukemia. Injections were given every 12 hours and blood analyses were made usually at 6 hours following injection. Blood analyses for these mice were continued throughout life and are explained further in the text.

(Fig. 1 and 2).

Degenerating lymphocytes both mature and immature were observed in peripheral blood 6 hours after initial injection of adrenal cortical hormone and constituted as much as 20% of the differential count in several leukemic animals which had received injections over a period of 10 days to 2 weeks or longer. Nuclear degenerative changes were prominent in these cells. Dougherty and White[2] have not described a peripheral removal of lymphocytes in their experimental observations in normal mice but suggest this as a possible mechanism in normal lymphocyte dissolution. Whether the phenomenon observed here is characteristic of the lymphocytes of leukemic mice or is related to a strain difference in the mice employed must await further experimentation.

Favorable effects of adrenal cortical extract on hemoglobin levels in leukemic mice have not been observed, although it is possible that the decline in hemoglobin levels is not as precipitous in treated as in control leukemic animals.

Profound palliative effects were noted within 24 to 48 hours in the majority of leukemic mice. These effects were: complete disappearance of the symptoms of dyspnea and thoracic enlargement and marked regression in infiltrated inguinal, axillary, cervical and mesenteric lymph-nodes. Definite regression of the spleen was noted in some leukemic animals but this was not constant throughout the treated series. It has been reported in normal mice receiving injections of adrenotropic hormone that the spleen did not show the characteristic weight decrease observed for other lymphoid tissues.[3] The characteristic response to adrenal cortical extract in a typical case, ♀ 10130 RIL, was as follows: Sixteen days after appearance of symptoms of leukemia this animal was in the terminal stages of leukemia. There was extreme dyspnea, moderate involvement of all subcutaneous lymph nodes, each measuring approximately 3 x 3

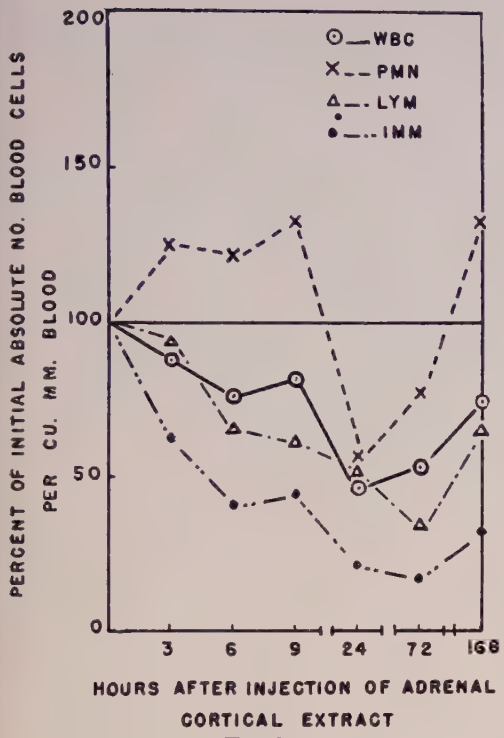

FIG. 2.

Mean percentage increase or decrease of initial number of blood cells in leukemic mice receiving daily injections of adrenal cortical extract. Each point on curve represents the mean of data obtained from at least 10 mice. Mean percentage increase at 168 hours is the result of including data of 2 mice which became refractory at this time.

mm, a moderately involved spleen and palpable mesenteric nodes. Biopsy tissue from an inguinal lymph node showed severe infiltration of immature forms and mice of the RIL strain inoculated with the remaining tissue of the inguinal node developed leukemia within 3 to 4 weeks. The leukocyte count was elevated to 40,500 and 24% of the circulating cells were lymphoblasts. Lipoadrenal cortex was injected subcutaneously 0.1 cc twice daily. Within 48 hours all subcutaneous nodes had completely regressed, there was no evidence of dyspnea and the spleen and mesenteric nodes showed definite regression. This animal continued to show regression with daily inoculation of extract and died 30 days after appearance of symptoms at which time the subcutaneous lymph nodes were normal in size, the thymus and spleen showed slight to moderate infiltration and the liver and kidneys were greatly infiltrated. (See Fig. 1 for blood response in this animal.)

Lymph nodes, spleen, thymuses, liver and other organs were removed following death of the animal for histological study. In addition several inguinal nodes were removed at 2 and 3 days following initial injection. Severe alterations in infiltrated lymph-nodes and thymuses were observed in leukemic mice which had received numerous injections of adrenal cortical extract. Extensive degenerative changes of the immature lymphocytes were evident. Nuclei were pyknotic and of various sizes and bizarre shapes. Hyperplasia of the cytoplasm was marked in numerous immature forms. Nuclear debris was scattered throughout the organ with very little evidence of phagocytosis. There was a severe depletion of immature forms and a "washed-out" appearance of nodes and thymuses. Recovery and repair processes were not noticeable. Mitotic figures were not observed. In contrast, the alterations in the spleen of leukemic mice receiving long continued injections were more localized. Numerous macrophages were present, filled with nuclear debris and there were many areas of histiocytic infiltration wherein the histiocyte-like cells had abundant cytoplasm but did not exhibit phagocytosis.

In inguinal lymph nodes examined at 2 and 3 days there were in evidence areas of recovery with many macrophages present, filled with nuclear debris. In addition in scattered areas there were present large reticular cells lying free in the sinuses but not actively phagocytic.

Degenerative changes of the immature lymphocytes were not found in the non-hemopoietic tissues studied.

Definite weight decreases of spleen, thymus and subcutaneous lymph-nodes of treated leukemic mice were obtained. The effect on thymic mass was most severe resulting in a mean weight of 270 $\pm$ 90 mg in the experimental series compared with 663 $\pm$ 66.5 mg in leukemic controls.

From the small number of mice observed in this preliminary series, it is impossible to state the effect on life expectancy. Two leukemic mice of the RIL strain have lived 30

and 42 days respectively with continued daily intraperitoneal inoculations. We have observed the course of the disease in more than 50 leukemic animals of this strain and none has survived for this period of time.

Summary. Injections of adrenal cortical extract into 13 mice of the inbred C58 and RIL strains in the terminal stages of spontaneous lymphoid leukemia resulted in the following responses: (a) An acute response (maximum at 6 hours following initial injection) resulting in a decrease of circulating leukocytes of the blood, a lymphopenia with a corresponding absolute polymorphonuclear leukocytosis and a decrease in the number of circulating immature lymphocytes. (b) These blood alterations become more pronounced following continuous daily injections and tend not to return to the leukemic blood picture within 24 hours after injection of the extract. (c) Regression of infiltrated thymuses and subcutaneous lymph nodes was observed. Regression of the spleen, although definite, was not so pronounced as that observed in other lymphoid tissues. (d) Extensive degenerative changes in immature lymphocytes in thymuses and lymph nodes resulting in pyknosis, dissolution and depletion of these cells. In the spleen similar changes occurred but were not so generalized. Pronounced degenerative changes in immature lymphocytes was observed in thymuses and lymph nodes of mice receiving daily injections over a relatively long period of time. Recovery and repair in these lymphoid organs was slight.

16046

Intertransformability of *Salmonella simsbury* and *Salmonella senftenberg.**

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It is recognized[1-7] that profound changes may be induced in H antigens of *Salmonella* by cultivating the organisms in serums containing appropriate H agglutinins. Such changes seem to occur particularly in monophasic cultures. Thus, in the stools of a person infected with *S. cholerae-suis* var. *kunzendorf* (VI,VII:1,5) Kristensen and Bojlen[8] found an organism with the biochemical properties of Kunzendorf whose antigens were VI,VII:c. Such variants are easily produced *in vitro* by cultivating Kunzendorf cultures in 1,5 serum. The writers have encountered 12 of these variants occurring naturally among cultures from man and swine. Likewise, Edwards and Moran[7] found that monophasic strains of *S. minnesota* (XXI,XXVI:b) were easily changed to a form with new H antigens (XXI,XXVI:z_{33}) by cultivation in b serum. Cultures with the latter formula occurred naturally among strains isolated from sewage by A. A. Hajna and it seems very

* The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director. It was supported in part by a research grant from the U. S. Public Health Service.

1 Kauffmann, F., *Z. Hyg.*, 1936, **119**, 104.

2 Gnosspelius, A., *Z. Hyg.*, 1939, **121**, 528.

3 Kauffmann, F., and Tesdal, M., *Z. Hyg.*, 1937, **120**, 168.

4 Edwards, P. R., and Bruner, D. W., *J. Bact.*, 1939, **38**, 63.

5 Bruner, D. W., and Edwards, P. R., *J. Bact.*, 1941, **42**, 467.

6 Edwards, P. R., and Bruner, D. W., *J. Bact.*, 1942, **44**, 289.

7 Edwards, P. R., and Moran, A. B., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 242.

8 Kristensen, M., and Bojlen, K., *Zbt. f. Bakt.*, I, Orig., 1936, **136**, 295.

likely that they arose from XXI,XXVI:b cultures by induced variation which occurred in nature.

Observations like the above cast suspicion on *Salmonella* types with unusual H antigens.

A type with hitherto unrecognized H antigens may be a derivative of some known type whose antigens have been changed. Bruner and Edwards[9] described *S. simsbury* (I,III,XIX:z_{27}) as a new type with distinct H antigens. Subsequently 10 additional representatives of *S. simsbury* were recognized among cultures received for typing. While the organism seemed to constitute a valid *Salmonella* type and was so accepted, the observations on *S. minnesota* indicated the need for further study of the relationship of *S. simsbury* to *S. senftenberg* (I,III,XIX:g,s,t).

Nine cultures of *S. simsbury* were plated and single colonies selected. The single colony isolations were examined and found to have O and H antigens characteristic of the type. They were inoculated into semisolid agar to which had been added z_{27} serum freed of O agglutinins by absorption with boiled cultures of *S. senftenberg*. One culture gave evidence of change in the H antigens by spreading slightly in the first transfer in serum-semisolid medium. From this spreading growth a form indistinguishable from *S. senftenberg* was isolated. Similar changes were observed in the remaining 8 strains of *S. simsbury* after 6 to 11 transfers in the medium.

The cultures of *S. simsbury* which had been changed to I,III,XIX:g,s,t and 10 typical cultures of *S. senftenberg* were then planted in semisolid medium containing g,s,t serum. These cultures proved more resistant to change than did the original *S. simsbury* strains. After 10 to 12 serial transfers over a period of 2 months they began to spread very slowly through the medium. After several additional transfers a form which had H antigens unlike any known *Salmonella* type was isolated from all the cultures. To this form the symbol z_{34} was applied.

The z_{34} forms were then transferred serially in semisolid medium which contained both g,s,t and z_{34} serums. After numerous transfers over a period of 3 months one of the *S. senftenberg* cultures spread rapidly through the medium. From this culture a form having the antigens I,III,XIX:z_{27} was isolated. The remaining z_{34} cultures gave no evidence of change.

The agglutinative characteristics of the various forms are given in Table I. Absorption tests confirmed the results obtained by agglutination. The g,s,t form of *S. simsbury* removed all agglutinins from *S. senftenberg* serum. Likewise, the z_{27} form of *S. senftenberg* exhausted *S. simsbury* serum. The changes brought about may be summarized as follows:

I,III,XIX:z_{27} ⟶ I,III,XIX:g,s,t
I,III,XIX:g,s,t ⟶ I,III,XIXz_{34} ⟶ I,III,XIX:z_{27}

S. simsbury and *S. senftenberg* must have descended from the same ancestral stock. Possibly this ancestor was a typical diphasic *Salmonella* which shifted normally between g,s,t and z_{27} phases. A stock culture of *S. simsbury* that had been transferred regularly on agar slants for 5 years was found to possess H antigens like those of *S. senftenberg*. The conditions under which the culture was kept made it highly improbable that it was mislabelled. This apparent spontaneous change of a stock culture from z_{27} to g,s,t supports the hypothesis that the ancestor of the two types was diphasic. A second possibility is that the parent culture was a more complex monophasic strain which spontaneously divided into two simpler components. Loss variation of this sort occurs in *S. hormaechei* (XXIX,Vi:z_{30},z_{31}) which produces XXIX,Vi:z_{30} and XXIX,Vi:z_{31} variants which are quite stable but which can be changed each into the other by growth in appropriate serums (Edwards[10]). A third possibility is that the very rare *S. simsbury* is an induced variant of the commonly occurring *S. senftenberg* just as XXI,XXVI:z_{33} seems to be an induced variant of XXI,XXVI:b. The difficulty of the g,s,t⟶z_{27} variation does not support this view.

It is not possible to state exactly how

[9] Bruner, D. W., and Edwards, P. R., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 174.

[10] Edwards, P. R., *J. Bact.*, 1946, **51**, 523.

TABLE I.
Agglutination and Absorption Tests.

Serums	Antigens					
	Senftenberg (g,s,t)	Senftenberg (z_{34})	Senftenberg (z_{27})	Simsbury (z_{27})	Simsbury (g,s,t)	Simsbury (z_{34})
Senftenberg (g,s,t)						
Unabsorbed	20000	0	500	500	20000	0
Absorbed by						
Simsbury (g,s,t)	0	0	0	0	0	0
Senftenberg (z_{34})	10000	0	500	500	10000	0
Senftenberg (z_{27})	10000	0	0	0	10000	0
Simsbury (z_{27})						
Unabsorbed	0	0	10000	10000	0	0
Absorbed by						
Senftenberg (z_{27})	0	0	0	0	0	0
Simsbury (z_{34})	0	0	10000	10000	0	0
Simsbury (g,s,t)	0	0	10000	10000	0	0
Senftenberg (z_{34})						
Unabsorbed	0	5000	500	500	0	2000
Absorbed by						
Senftenberg (g,s,t)	0	2000	500	500	0	2000
Simsbury (z_{34})	0	200	0	0	0	0
Simsbury (z_{34})						
Unabsorbed	0	5000	500	500	0	5000
Absorbed by						
Simsbury (z_{27})	0	5000	0	0	0	5000
Senftenberg (z_{34})	0	0	0	0	0	200

Figures indicate highest dilutions at which agglutination occurred.
0 indicates no agglutination at 1 to 200.

S. simsbury arose but since it obviously came from the same ancestor as *S. senftenberg*, it seems logical to omit *S. simsbury* from the Kauffmann-White classification and to assign *S. senftenberg* the formula I,III,XIX:g,s,t—z_{27}.

Summary. By growth in serums it was possible to change *S. simsbury* (I,III,XIX:z_{27}) into *S. senftenberg* (I,III,XIX:g,s,t) and vice-versa. The $z_{27} \longrightarrow$ g,s,t change was accomplished without difficulty but the reverse was done only by first transforming the H antigens to a hitherto unrecognized form (z_{34}) and then changing them to z_{27}. It is recommended that *S. simsbury* be dropped from the classification and that the formula of *S. senftenberg* be written I,III,XIX:g,s,t—z_{27}.

16047

Hemoprotein from Root Nodules and Nitrogen Fixation by *Rhizobium*.*

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Although the necessity of root nodule bacteria for fixation of molecular nitrogen by leguminous plants has been established since 1886, experimental proof that these organisms are the responsible agent still is lacking. In spite of numerous claims of fixation by pure cultures of the bacteria, few such claims withstand critical examination.[1] The identification of the red pigment in root nodules as a hemoprotein[2-5] led inevitably to the suggestion it was directly concerned with the fixation reaction. Among the support for this proposal was an experiment by Virtanen and Laine[6] in which addition of nodular extracts to pure cultures of *Rhizobium* apparently enabled them to fix appreciable quantities of nitrogen. The fixation was particularly striking if oxalacetic acid was added with the extract. This claim appeared surprising in view of the difficulty in inducing excised nodules to assimilate molecular nitrogen although they still contain both bacteria and pigment. Nevertheless, because of its obvious importance, extensive tests of the possibility were undertaken. Such tests are limited to the summer months when sufficient nodules can be grown to provide pigment for extensive replication. During the past 2 summers we have made these tests but have obtained no evidence that the presence of the pigment or any other constituent of the extract of nodules from soybean or pea stimulate the free living *Rhizobium* to fix nitrogen. Since these experiments were completed, a new report from Virtanen[7] states that more extensive trials fail to confirm their first experiment; we shall summarize here only the experimental variations tested and the results.

Materials and Methods. Nodules were taken from leguminous plants grown on a nitrogen-poor sand in a cold-frame. Twice during the growing season the plants were watered with Hoagland's N-free nutrient solution. The nodules were picked into cold water, drained, and macerated with an equal quantity of water. The extract was pressed through cheesecloth, centrifuged for 20 minutes, then passed through a Berkefeld *N* filter and suitable aliquots added to 6 oz prescription bottles containing 25 ml N-free medium. This medium was: Allison's salt mixture, 1.5 g; sucrose, 10 g; water, 1000 ml. Traces of Fe and Mo were supplied together with 1 ml/liter yeast water (2 mg N) for growth factors. When the soybean organism was used, 1 ml of a mesquite gum hydrolysate was also added. Various species of *Rhizobium* were grown on agar slants (Medium 79 of Fred and Waksman), and aliquots of their suspensions added to the N-free medium. The incubation was at 30°C for 7 days; total nitrogen was determined by a semimicro Kjeldahl procedure sensitive to 0.02 mg.

Experimental variations tested in attempts to secure fixation included:

1. *Source of nodules.* Soybeans inoculated with *Rhizobium japonicum* 534 and Canada field peas with *R. leguminosarum* 317 were used. Nodules from plants 6 to 9 weeks old

* Supported in part by grants from the Rockefeller Foundation and from the Research Committee of the Graduate School from funds provided by the Wisconsin Alumni Research Foundation.

1 Wilson, P. W., *The Biochemistry of Symbiotic Nitrogen Fixation*, University of Wisconsin Press, 1940.

2 Kubo, H., *Acta Phytochim.* (Japan), 1939, **11**, 195.

3 Burris, R. H., and Haas, E., *J. Biol. Chem.*, 1944, **155**, 227.

4 Keilin, D., and Wang, Y. L., *Nature*, 1945, **155**, 227.

5 Virtanen, A. I., *Nature*, 1945, **155**, 747.

6 Virtanen, A. I., and Laine, T., *Suomen Kemistilehti*, 1945, **18**B, 39.

7 Virtanen, A. I., Jorma, J., Linkola, H., and Linnasalma, A., *Acta Chemica Scand.*, 1947, **1**, 90.

TABLE I.
Summary of Nitrogen Fixation Experiments.

Treatment	Exp. I		Exp. II		Exp. III	
	No. of samples	Mean N, mg/25 ml	No. of samples	Mean N, mg/25 ml	No. of samples	Mean N, mg/25 ml
Control	2	2.95	2	2.57	2	2.65
None	2	2.96	3	2.62	3	2.63
α-Ketoglutarate	2	2.96	4	2.51	4	2.72
Oxalacetate	2	2.99	3	2.60	4	2.64
Citrate	2	3.03	4	2.66	3	2.73
	Exp. IV		Exp. V		Exp. VI	
Control	3	3.25	2	1.63	2	3.38
None	4	3.24	—	—	—	—
α-Ketoglutarate	3	3.25	2	1.66	2	3.27
Oxalacetate	2	6.37*	2	1.38	3	3.73
Citrate	3	3.19	2	1.69	2	3.25
	4	6.38*				
	Exp. VII		Exp. VIII		Exp. IX	
Control	2	2.41	2	3.20	2	2.64
None	2	2.49	2	3.19	3	2.91
α-Ketoglutarate	4	2.43	3	3.36	2	2.99
Oxalacetate	3	2.38	3	3.22	—	—
Citrate	2	2.45	3	6.14*	2	2.94

Extract of nodules containing pigment added to all samples; those marked with asterisk given twice the level of others. Controls kept at 3°C, others at 30°C.

gave the most satisfactory extracts.

2. *Method of Extraction.* In about one-half the experiments, the nodules were extracted in the cold under carbon monoxide. Before its addition to the medium the pigment was reoxygenated for 20 minutes.

3. *Filtration.* The finest filter tried was a Seitz, but a Berkefeld *N* was usually used as it did not clog. Extracts of nodules from plants in the flowering stage were so viscous that they passed only through the cheesecloth.

4. *Organism.* Species of *Rhizobium* tested, singly or in mixtures were *R. japonicum, R. leguminosarum, R. melilotii,* and *R. trifolii.*

5. *Additions.* Various organic acids, oxalacetic, α-ketoglutaric and citric, often implicated in fixation schemes were added (15 mg/25 ml).

6. *Incubation.* In some experiments the bacterial cells were incubated for 3 days in the N-free medium before the addition of the extract as was suggested by Virtanen and Laine;[6] in others, the cells and extract were added at the same time.

7. *Miscellaneous.* Two to 4 replicates were made of each treatment. The controls were treated in the same way as the experimental flasks but were kept at 3°C. The quantity of pigment added varied with the preparation, but an attempt was made to standardize this so that the extract added to 25 ml contained 2 to 3 mg N. In 2 experiments twice the usual level of extract was supplied. About 0.1-0.2 mg N was contained in the bacteria used as inoculum.

Results. The agreement in nitrogen content among replicates is illustrated by the data from a typical experiment: Control, 2.60, 2.53; plus extract, 2.62, 2.61, 2.63; plus extract and oxalacetate, 2.58, 2.58, 2.62; plus extract and α-ketoglutarate, 2.66, 2.50, 2.63, 2.62; plus extract and citrate, 2.55, 2.72, 2.58, 2.69 mg per 25 ml. Statistical analysis of the data from 9 experiments (113 samples) indicated that the standard deviation of the means of duplicates was about 0.15 mg and of the means of quadruplicates about 0.10 mg. Therefore, the means of the replicates for each treatment would have to exceed that of the control by 0.2 to 0.3 mg even to approach statistical significance. Actually, of course, one would wish greater differences for so important a conclusion, *e.g.*, a gain of at least 0.5 to 1 mg of N per bottle. As can be seen

by the summary in Table I no such gains were obtained. It is concluded that if fixation occurs it is too weak to be detected by the Kjeldahl method. The possibility that the slight gains occasionally observed possess significance is now being tested using the much more sensitive isotopic method.

Summary. Fixation of molecular nitrogen could not be induced in free-living cultures of *Rhizobium* by supplying them with extracts of root nodules containing the hemoglobin-like pigment. Nine experiments were made during 2 growing seasons. Variations in technique included: species of bacteria; source of nodular extract; addition of oxalacetic, α-ketoglutaric and citric acids; and method of preparing the extract. Analyses of the results showed that gains of 0.2-0.3 mg N were required for statistical significance, but even these slight increases were not consistently obtained.

16048

Effect of Induced Liver Cirrhosis on the Reproductive System of the Male Rat.*

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While it has been shown repeatedly that the physiology of reproduction is dependent upon the maintenance of a normal pituitary-gonadal balance, it is also becoming increasingly evident that intact liver function is essential to the normal metabolism of the sex steroid hormones. The results of *in vitro* experiments performed by numerous investigators have provided ample evidence that the liver is the principal organ responsible for the inactivation and removal from the blood of the natural estrogen of the body (Silberstein *et al.*,[1] Zondek,[2] Engel and Navratel,[3] Heller *et al.*,[4] Heller,[5] and Engel,[6]). Inactivation of endogenous estrogens by the liver has been reported by numerous workers using varying techniques. Talbot[7] induced liver damage in female rats with carbon tetrachloride in alcohol and found an increase in uterine weight of 200% by the third day of the experiment. From this he concluded that the poisoned animals were exposed to an increased concentration of blood estrogen because of the impaired inactivating capacity of the liver.

Other experiments (Golden and Severinghaus,[8] Biskind and Mark,[9] Biskind,[10] Krichesky, Benjamin and Slater,[11]) were devised to divert the gonadal hormones directly into the portal or into the systemic circulation. When the portal route was used the hormones were promptly inactivated, as shown by failure to maintain the sex accessories of castrate animals, whereas the hormones escaped inactivation when they drained directly into

* Aided by grants from Ayerst, McKenna, and Harrison, Ltd., Rouses Point, New York, and the Board of Research, University of California.

1 Silberstein, F., Engel, P., and Molnar, K., *Klin. Wchnshr.*, 1933, **12**, 1693.

2 Zondek, B., *Skand. Arch. fur Physiol.*, 1934, **70**, 133.

3 Engel, P., and Navratel, E., *Biochem. Z.*, 1937, **292**, 434.

4 Heller, C. G., Heller, E. J., and Severinghaus, E. L., *Am. J. Physiol.*, 1939, **126**, 530.

5 Heller, C. G., *Endocrin.*, 1940, **26**, 619.

6 Engel, P., *Endocrin.*, 1941, **29**, 290.

7 Talbot, N. B., *Endocrin.*, 1941, **25**, 290.

8 Golden, J. B., and Severinghaus, E. L., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 361.

9 Biskind, G. R., and Mark, J., *Bull. Johns Hopkins Hosp.*, 1939, **65**, 212.

10 Biskind, G. R., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 266.

11 Krichesky, B., Benjamin, J. A., and Slater, C., *Endocrin.*, 1943, **32**, 345.

the systemic circulation and consequently the sex accessories were maintained. The usual method was to transplant the gonad into the mesentery to facilitate drainage into the portal vein or to transplant the gonad into the axilla or body wall to prevent portal drainage. Steroid hormone pellet implants in the spleen of castrate rats was the method favored by Biskind and his associates.

Further indication of hepatic inactivation is provided by the study of steroid hormone metabolism in the presence of experimental or clinical liver damage. Extensive liver disease seems to impair hormone inactivation so that estrogenic effects are usually intensified Pincus and Martin[12] report an 80% increase in estrogen effectiveness in rats with experimental liver damage. Human observations (Edmondson *et al.*,[13] Glass, *et al.*,[14,15] Gilder and Hoagland,[16] and others) imply that acute or chronic liver disease, if extensive enough, may be associated with impairment of steroid hormone metabolism. Such disease may be followed by impairment of testicular function or the development of the full blown syndrome of testicular atrophy, gynecomastia and torso alopecia. The latter syndrome has been ascribed by one of us (Glass) to the effects of circulating biologically active estrogens emanating from failure of hepatic inactivation. To test this thesis it was deemed advisable to study the sex organs of male rats with experimental liver damage. The spontaneous effects of liver damage on the sex organs of the experimental animal have not been adequately investigated.

Method. Eighty-four mature male Wistar albino rats weighing from 150 to 210 g were used and were maintained on Rockland rat pellets and lettuce. All animals were intubated thrice weekly and given various amounts of carbon tetrachloride in 50% alcohol. Two experiments were carried out.

Experiment I. A pilot experiment using 35 animals was undertaken to determine the amount of carbon tetrachloride that would produce maximum hepatic damage with minimum mortality. The animals were divided into 3 approximately equal groups: Group A received 0.025 cc carbon tetrachloride in alcohol in doses of 0.5 cc; Group B received 0.05 cc in doses of 0.5 cc; Group C received 0.10 cc in doses of 1.0 cc. Animals from each group were sacrificed at selected intervals. Biopsies were carried out during the course of the treatment which continued for as long as 12 weeks in some animals. Macroscopic examination of the liver was made at autopsy and portions of the liver and kidney were prepared for histologic study.

Experiment II. Having determined an adequate dosage of carbon tetrachloride, a second experiment was carried out to determine the effects of hepatic injury on the male reproductive system. Forty-nine animals were used, 17 either died or were sacrificed for histologic material during the course of the experiment and the remaining 32 provided the quantitative data. The experimental animals were intubated 3 times per week with 0.05 cc carbon tetrachloride in 50% alcohol solution in doses of 0.5 cc. Five groups were employed: Group 1, animals serving as controls without treatment; Group 2, animals intubated over a period of 22 days; Group 3, animals intubated over a period of 43 days; Group 4, animals intubated over 60 to 80 days; Group 5, animals intubated for 88 days and then maintained without treatment for 40 days.

All animals were weighed before and after the experiment. The sex accessories were carefully dissected free of surrounding connective tissues and were weighed on a torsion balance. The testes were also removed and weighed. These structures and the liver and kidneys were preserved for histologic study.

Results. In the first experiment the mortality rate was greatest in those animals receiving the largest dose of carbon tetrachloride. Within a few days they appeared sickly; the

[12] Pincus, G., and Martin, D. W., *Endocrin.*, 1940, **27**, 838.

[13] Edmondson, H. A., Glass, S. J., and Soll, S. N., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 97.

[14] Glass, S. J., Edmondson, H. A., and Soll, S. N., *Endocrin.*, 1940, **27**, 749.

[15] Glass, S. J., Edmondson, H. A., and Soll, S. N., *J. Clin. Endocrin.*, 1944, **4**, 54.

[16] Gilder, H., and Hoagland, C. L., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 62.

TABLE I.
Effect of Carbon Tetrachloride Intubation of the Weight of the Sex Accessories in the Male Rat (0.05 cc CCl_4 in 50% Alcohol Administered 3 × Weekly).

Group No.	No. of animals	Duration of treatment, days	Avg body wt, g	Avg sex acces. wt, mg	Avg testis wt, mg
1	8	0	163	845	1841
2	6	22	160	637	1921
3	7	43	154	390	1663
4	7	60-80	155	278	1840
5*	4	88+40	207	476	1586

* In Group 5 carbon tetrachloride was administered for 88 days. The animals were then maintained for 40 days without treatment before they were sacrificed.

fur was rough; the backs were arched and they lost weight rapidly. Most of these were sacrificed in order to obtain fresh tissues for histologic study. The mortality among the other 2 groups was almost the same (approximately 20%) and only few of the surviving animals showed observable evidence of illness. Liver necrosis appeared in the animals receiving 0.025 cc of carbon tetrachloride by the 19th day and by the 11th day in the 0.05 cc group. Cirrhosis was present in the 0.025 cc group by the 58th day and by the 44th day in 0.05 cc group. The latter dose, therefore, produced maximum hepatic injury in the shortest time with no greater mortality than in the .025 cc group.

The liver manifested a series of progressive architectural changes not unlike that seen in human cirrhosis, that is, cloudy swelling and fatty infiltration in early stages, followed by central necrosis of the lobule with attempts at healing in the longer term experiments. Fibrosis, as well as gross nodularity was evident after 44 days of carbon tetrachloride feeding. In animals with more advanced liver failure, ascites was usually present.

From the onset of carbon tetrachloride intubation the kidneys of all animals became pale in color and later took on a yellowish cast. In the long term experiments the color was usually a chocolate brown. The histologic picture was remarkably constant with an early cloudy or albuminoid swelling followed by more severe swelling of the cells and accompanied by a coarsening of the reticulum and a clouding or granulation of the cytoplasm. This persisted throughout the experiment and was nearly constant at all dosage levels.

In the second experiment the pertinent data are given in Table I. The average weight of the sex accessories (including seminal vesicles, coagulating gland, ampullary gland, and the lateral, dorsal and ventral lobes of the prostate) was 845 mg in the untreated animals. After 22 days of treatment with 0.05 cc carbon tetrachloride the average weight of the accessories was 637 mg or approximately a 25% decrease. After 43 days of treatment the average weight was decreased by 53% to 390 mg. The group receiving treatment for 60 to 80 days showed accessories weighing only 278 mg, a 67% decrease below the untreated controls. The final group treated for 88 days and then maintained for 40 days without treatment before being sacrificed showed partial recovery in the weight of the accessories. In this group the accessories averaged 476 mg, a decrease of 44% below the controls and an increase of 70% over the group treated for 60 to 80 days and sacrificed immediately thereafter.

Although there was striking atrophy of the sex accessories following liver damage by carbon tetrachloride, yet the results indicate that the testicles were not so greatly affected. There was great variation in testicular weight among animals of all groups and because the number of animals used was small the data given in Table I are not considered significant.

Histologic examination of the testes revealed no striking architectural disorganization of the seminiferous tubules. Spermatogenesis was not greatly impaired in the majority of animals. In only 2 of 32 testes examined was marked testicular damage observed. This was indicated by absence of mature sperm and marked atrophy of spermatogenic cells.

Discussion. The data presented indicate that the oral administration of carbon tetrachloride produces hepatic damage and results in atrophy of the secondary sex organs of the male rat as indicated by reduction of weight part of which may be accounted for by the atrophy of bled tissue and part by possible reduction in the amount of the secretions of these organs. This treatment causes no significant weight changes in the testes. Healing of the liver damage promptly follows withdrawal of the poisoning agent and is accompanied by partial recovery of the weight of sex accessory organs 40 days after cessation of carbon tetrachloride administration.

In explaining these data two possible hypotheses suggest themselves; (1) that hepatic damage and sex accessory atrophy may be produced by the toxicity of the carbon tetrachloride and (2) that liver damage due to carbon tetrachloride may cause failure of hepatic inactivation of endogenous estrogens resulting in an increased level of circulating estrogens.

In the first instance, it must be admitted that direct toxic effects of carbon tetrachloride on the accessories have not been eliminated in these experiments. The atrophy of the secondary sex organs may well be due to a direct toxic action of the poison in a manner similar to the damage produced in the liver. However, it would be remarkable that such toxicity would affect only the accessory organs and not the testes, especially since the latter have been reported to be highly sensitive to noxious agents. It is surprising that only 2 animals of 32 examined show degenerative changes in the testes and no significant weight loss. The accessory organs, on the other hand, in all animals, exhibit a weight loss ranging from 25 to 67% below untreated animals. If this atrophy is due to a direct toxicity of carbon tetrachloride, then these data suggest that in the doses employed, the poison affects the sex accessory organs preferentially and has little or no effect on the testes. A difference in threshold of these organs obtains some support from the findings of Simpson and Evans[17] that the sex accessories are less resistant to androgen deprivation than the testicular tubules.

It should be pointed out also that in some unpublished experiments carried out in this laboratory in which hepatic damage was induced in rats by a low protein diet (4% casein with vitamin supplements), atrophy of the prostate and seminal vesicles was observed in the males. These results, without the use of a toxic agent, were qualitatively similar to those after carbon tetrachloride feeding. It should be recognized, however, that deficiency diets, by interference with normal metabolism, may serve as toxic agents in themselves.

A second possible explanation, that liver damage induced by carbon tetrachloride feeding may prevent hepatic inactivation of endogenous estrogens resulting in an increased level of circulating estrogens, may account adequately for the results reported here.† A high level of circulating estrogens, especially in the "free" form as suggested by Glass, Edmundson and Soll may induce secondary sex atrophy either by a direct inhibition of these organs or by inhibition of pituitary gonadotropin secretion. This view is supported by the findings of Morrione[18] that doses of estrogens insufficient to produce testicular damage in normal rats resulted in severe testicular damage when given to animals with livers damaged by carbon tetrachloride feeding. He suggested that these low doses were effective only in the latter group of animals (with damaged livers) because circulating estrogen levels in them were increased.

Healing of liver injury and recovery of the sex accessories following withdrawal of carbon tetrachloride feeding may be explained by either hypothesis. Withdrawal of the poison

[17] Simpson, M. E., Li, C. H., and Evans, H. M., *Endocrin.*, 1944, **35**, 96.

† That circulating endogenous estrogens after hepatic injury are increased is supported by unpublished data from this laboratory in which bioassay showed approximately a thousand-fold increase in urinary estrogens excreted by female guinea pigs with carbon tetrachloride-damaged livers over that excreted by normal animals.

[18] Morrione, T. G., *Arch. Path.*, 1944, **37**, 39.

permits healing and recovery by removing the deleterious agent or by healing of the liver and a return to normal steroid metabolism with consequent inactivation of endogenous estrogens.

Summary. Pathologic changes in the liver and kidneys of male rats fed carbon tetrachloride is described. Striking atrophy of the secondary sex organs but no significant changes in weight of testes follows variable periods of carbon tetrachloride feeding. Withdrawal of the poisonous agent results in healing of the liver and partial weight recovery of the sex accessories.

16049 P

Catheterization of the Coronary Sinus and the Middle Cardiac Vein in Man.*

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A technique of intravenous catheterization of the coronary sinus has been developed recently in intact dogs by Goodale and Lubin,[1] using the Cournand intravenous catheter.[2] This method has been employed by Eckenhoff and co-workers[3] to measure coronary blood flow, using the nitrous oxide method developed by Kety and Schmidt for the determination of cerebral blood flow.[4] The procedure appears to be less hazardous than the method in intact dogs previously reported by Harrison and co-workers,[5] which involved the use of a brass balloon cannula. In a combined study of 20 different dogs,[1,3] as many as 7 catheterizations and 4 duplicate measurements of coronary flow have been performed on the same dog at monthly intervals. These developments suggested that a similar procedure might be followed in the determination of coronary blood flow in man. This paper is a preliminary report of 9 successful catheterizations of the coronary vessels in man.

Methods. Most of the patients studied had congenital heart disease. Consequently, catheterization of the heart of these individuals was primarily undertaken to obtain information concerning the nature of the cardiac anomalies.[6,7,8] Only one subject, (No. 7, Table I), had a normal heart. For catheterization of the right heart the technique of Cournand[2] was followed, using standard No. 7 catheters. Passage of the catheter into the coronary sinus or the middle cardiac vein was verified by (a) fluoroscopic control, (b) recording of pressures, and (c) determinations of blood oxygen and carbon dioxide contents. It was assumed that the coronary sinus had been successfully intubated if the systolic pressures were below ventricular and above auricular systolic pres-

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† First Lt., Medical Corps, A.U.S., Army Chemical Center, Maryland.

1 Goodale, W. T., Lubin, M., and Banfield, W. G., in press.

2 Cournand, A., *Fed. Proc.*, 1945, **4**, 207.

3 Eckenhoff, J. E., Hafkenschiel, J. H., Harmel, M., Goodale, W. T., Lubin, M., and Kety, S. S., in press.

4 Kety, S. S., and Schmidt, C. F., *Am. J. Physiol.*, 1945, **143**, 53.

5 Harrison, T. R., Friedman, B., and Resnick, H., Jr., *Arch. Int. Med.*, 1936, **57**, 927.

6 Bing, R. J., Vandam, L. D., and Gray, F. D., Jr., *Bull. Johns Hopkins Hosp.*, 1947, **80**, 107.

7 Bing, R. J., Vandam, L. D., and Gray, F. D., Jr., *Bull. Johns Hopkins Hosp.*, 1947, **80**, 121.

8 Bing, R. J., Vandam, L. D., and Gray, F. D., Jr., *Bull. Johns Hopkins Hosp.*, 1947, **80**, 323.

TABLE I.
Blood Gas Values Obtained from Catheterization of Heart and Coronary Sinus.

No.	Date	Age, yrs	Sex	O_2 content R.A. vol. %	O_2 content R.V. vol. %	O_2 content C.S. vol. %	O_2 content F.A. vol. %	A-V oxygen difference: Coronary F.A.-C.S.	A-V oxygen difference: Systemic F.A.-Rt. Heart
1.	5/31/46	16	M	18.8	18.5	6.7	22.9	16.2	4.4
2.	7/ 2	16	M	17.0	18.0	8.8	22.0	13.2	5.0
3.	26	17	F	18.1	17.8	8.3	24.6	16.3	6.8
4.	31	16	M	24.0	24.9	9.5	28.2	18.7	4.2
5.	8/ 5	14	F	15.2	17.3	6.3	21.2	14.9	5.0
6.	2/14/47	19	F	9.5	12.6	5.5	16.8	11.3	7.3
7.	5/ 2	23	F	—	13.4	5.0	16.2	11.2	2.8
8.	20	11	F	14.9	14.8	5.2	17.6	12.4	2.8
9.	23	28	F	18.3	19.9	8.9	24.9	16.0	6.6

R.A.—Right auricle.
R.V.—Right ventricle.
C.S.—Coronary sinus.
F.A.—Femoral artery.

sures,[9] and if the oxygen contents of the sinus blood were significantly below those of returning mixed venous blood.[1] Under fluoroscopy the catheter enters the coronary sinus just below the region of the tricuspid valve, slightly medial, superior and anterior to the inferior vena cava. When the catheter is in the coronary sinus, it is seen curved upward toward the base of the heart. When the middle coronary vein is intubated, the catheter lies alongside the lower borders of the heart with the tip directed toward the apex. In the first five cases intubation of these vessels was fortuitous. A similar experience has been reported by others.[10] In the remaining 4 cases, catheterization of the sinus was carried out deliberately. Pressures were recorded with the Hamilton manometer.[11] All gas analyses were performed with the manometric method of Van Slyke and Neill.[12]

Results. Table I demonstrates that the oxygen contents of coronary venous blood were significantly below those of both right auricular and ventricular blood. The difference between the oxygen contents of peripheral arterial blood, (and consequently of coronary arterial blood), and of coronary venous blood varied from 11.3 to 18.7 vol %, while the total systemic arteriovenous oxygen difference varied from 2.8-7.3 vol %, (Table I). In three cases in which pressures were recorded, the average blood pressure in the coronary sinus was 12 mm Hg.

Summary. The coronary sinus and the middle cardiac vein of man has been successfully catheterized. Work is in progress to utilize this technique in the measurement of coronary blood flow in man.

9 Best and Taylor, 4th Edition, p. 278.

10 Dexter, L., and Sosman, M. C., *Radiology*, 1947, **48**, 441.

11 Hamilton, W. F., Broener, G., and Brotman, I., *Am. J. Physiol.*, 1934, **107**, 427.

12 Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

16050

Effects of Dibenamine on Cardiovascular Actions of Epinephrine, Acetylcholine, Pitressin and Angiotonin in Unanesthetized Dogs.*

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Nickerson and Goodman[1] have described adrenolytic actions of N, N-dibenzyl beta-chloroethyl amine (Dibenamine). They found that the compound, when injected slowly intravenously, produced minimal effects upon arterial blood pressure and, within 30 minutes after the injection, the pressor action of epinephrine was blocked in some species and reversed in others. The effects of stimulating various excitatory adrenergic nerves and the excitatory actions of epinephrine upon various effectors were prevented by Dibenamine. On the other hand, the inhibitory actions of epinephrine upon the non-pregnant cat uterus *in situ* and on the isolated small intestine of the rabbit and rat were not prevented by Dibenamine. They also observed that larger doses were required to block the effects of stimulating excitatory adrenergic nerves than were required to block the excitatory effects of injected epinephrine. Cardio-accelerator actions of epinephrine were not blocked by Dibenamine.

The present study is concerned principally with the cardiovascular actions of Dibenamine and with its effects upon the cardiovascular responses to epinephrine, acetylcholine, pitressin, and angiotonin in unanesthetized dogs.

Methods. Unanesthetized animals were trained to lie quietly on the table while a needle was kept in place in the radial vein. Heart rate was determined by counting the apex beat and from continuous electrocardiographic records taken during injection of the 4 test compounds. In one animal the spinal cord had been transected 24 hours previously and blood pressure was recorded from the femoral artery by the use of a mercury manometer.

N, N-dibenzyl beta-chloroethyl-amine hydro-chloride was prepared for injection by dissolving 200 mg in approximately 15 cc of propylene glycol, and this was diluted with an equal amount of water. Injection of the solution was started within 2 to 5 minutes after preparation, and a period of 5 to 20 minutes was required to give the animal the total dose of 200 mg.

Seven dogs weighing between 9 and 13 kg were used. Six of these were intact, and one had the spinal cord transected. The effects of test doses of epinephrine, acetylcholine, pitressin, and angiotonin upon heart rate were recorded before and at stated intervals after administration of Dibenamine. During each series of injections a needle was placed in the radial vein and kept open by injecting small amounts of isotonic saline. At the desired time a syringe containing the exact amount of the compound to be administered was substituted for the syringe containing saline and the compound was injected rapidly. Ample time was allowed for return of the heart rate to the resting level between injections.

Results. A. *Effects of Dibenamine on heart rate.* Dibenamine alone caused an increase in heart rate in each of the 6 intact animals. The maximal increase above the basal rate ranged from 46% to 95%, and it occurred in 10 to 25 minutes from the beginning of the injection. Usually the rates returned to the resting level within one to 3 hours.

B. *Effects of epinephrine on heart rate before and after Dibenamine.* Continuous electrocardiographic records were obtained during the test dose of epinephrine (2 cc of a 1-100,000 dilution) in 4 dogs before and after administration of Dibenamine. The averages of the rates for the 4 animals are graphed in Fig. 1.

The test dose of epinephrine produced a

* Aided by a grant from the John and Mary R. Markle Foundation.

1 Nickerson, M., and Goodman, L., *J. Pharm. and Exp. Therap.*, 1947, **89**, 167.

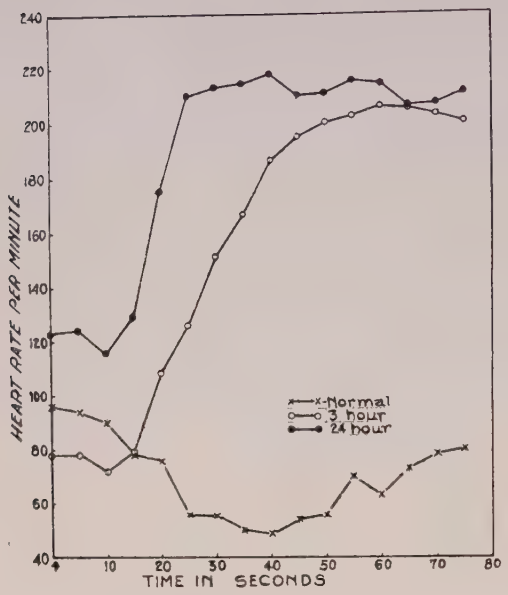

FIG. 1.

maximal decrease in rate of 45% to 57% in the 4 intact animals before Dibenamine. Since the direct influence of epinephrine upon the sino-auricular node is excitatory, this bradycardia is best explained on the basis of reflex effects elicited by a rise in blood pressure.

Three hours after Dibenamine had been administered the average heart rate was 19 beats per minute slower than the average of the control rates. At this time the test dose of epinephrine produced an acceleration of 97% to 208% above the rate preceding the injection. Acceleration was clearly evident within 20 seconds after the injection of epinephrine and progressed smoothly to a plateau at about 50 seconds. It was still maintained at 75 seconds. The effects of epinephrine on the heart rate 24 hours after Dibenamine were similar to the effects seen 3 hours after Dibenamine.

The reversal of the effect of the test dose of epinephrine on the heart rate of unanesthetized animals by Dibenamine may be readily explained if it is considered that epinephrine causes a fall in blood pressure in the unanesthetized animals after they have received Dibenamine. If such is the case, a smooth cardiac acceleration would be expected because of the combination of the direct accelerator influence of epinephrine and the reflex accelerator influence of the fall in blood pressure. This interpretation is supported by the observation that in an unanesthetized animal, with spinal cord transected at T_{12} so that blood pressure could be recorded from the femoral artery, injection of the test dose of epinephrine, after the animal had been given Dibenamine, caused an immediate fall of 50 mm of mercury in the mean arterial blood pressure. Animals under the influence of Dibenamine also characteristically showed much greater respiratory stimulation after the epinephrine injection than was seen in the unmedicated animals. Presumably, this respiratory stimulation is elicited reflexly from the fall in blood pressure.

The striking cardiac acceleration in response to epinephrine after Dibenamine would seem to indicate that the cardiac effects of epinephrine or of cardio-accelerator nerves are not blocked. However, a large part of this acceleration could be on the basis of decreased tonus of cholinergic cardio-inhibitory nerves.

C. *Effects of Dibenamine on the cardio-accelerator response to acetylcholine.* The brief hypotension produced by intravenous injection of acetylcholine elicits a typical cardio-accelerator response. The acceleration is due largely to reflex activation of adrenergic nerves and to liberation of epinephrine

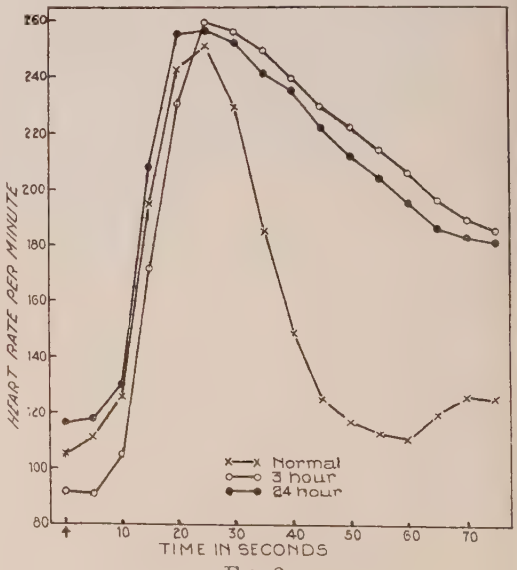

FIG. 2.

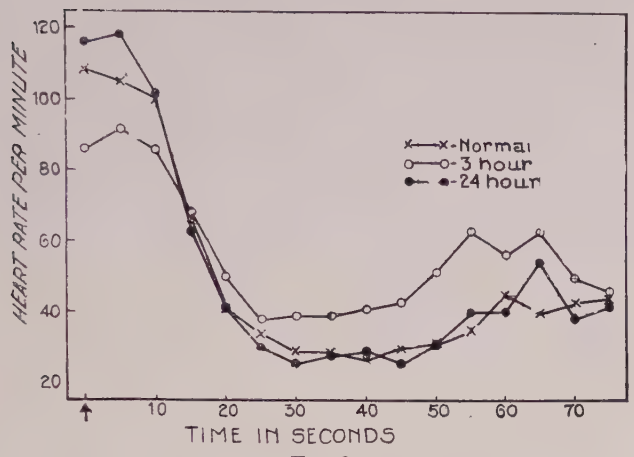

FIG. 3.

from the adrenal medulla.[2] Five animals were given a test dose of 1 mg of acetylcholine at 3 hours and again at 24 hours after the administration of Dibenamine. In Fig. 2 a curve is shown which illustrates the average of the cardio-accelerator responses to the test dose of acetylcholine in 23 animals. The other 2 curves in Fig. 2 illustrate the averages of the cardio-accelerator response to acetylcholine in 4 animals at 3 hours and at 24 hours after Dibenamine. The degree of acceleration was at least as great after Dibenamine as before. In both the normal animals and in those under the influence of Dibenamine, the maximum increase in heart rate in response to acetylcholine occurred at 25 seconds. In the normal animals the rate fell off rapidly, to reach a resting level within 50 to 60 seconds, but in the animals under Dibenamine the return to the resting level was quite delayed. The persistence of the fast rate would indicate that Dibenamine interferes with the restoration of the blood pressure to the normal level after a test dose of acetylcholine. This result is compatible with the interpretation that vasoconstrictor mechanisms are impaired by Dibenamine while cardio-accelerator mechanisms are relatively intact.

D. *Effects of Dibenamine upon the cardio-inhibitory response to pitressin and angiotonin.* The effects of Dibenamine on the pressor actions of pitressin and angiotonin were tested in 3 dogs which had received sodium pentobarbital. At the time when the pressor action of epinephrine was reversed, there was no interference with the pressor actions of either pitressin or angiotonin. In one animal under sodium pentobarbital, neurogenic hypertension was produced by sino-aortic denervation. When Dibenamine was injected in this animal, it produced a profound fall in blood pressure.

In unanesthetized dogs pitressin and angiotonin produce a pronounced reflex bradycardia during the rise in blood pressure resulting from their vasoconstrictor action.[3] If the vasoconstrictor action of these compounds is not blocked by Dibenamine, they would be expected to produce the typical cardio-inhibitory response. The effects of a test dose of 1½ pressor units of pitressin before and after Dibenamine were studied in 4 dogs, and the effects of a test dose of 20 pressor units of angiotonin were studied in 3 dogs. The averages of the cardio-inhibitory responses to pitressin in 4 dogs at 3 hours and at 24 hours after Dibenamine are shown in Fig. 3, and these are compared with a curve showing the averages of 14 normals. The results of a typical experiment with angiotonin are graphed in Fig. 4.

From these results it is evident that Dibena-

[2] Youmans, W. B., Aumann, K., Haney, H., and Wynia, F., *Am. J. Physiol.*, 1940, **128**, 467.

[3] Haney, H. F., Lindgren, A. J., Karstens, A. I., and Youmans, W. B., *Am. J. Physiol.*, 1943, **139**, 675.

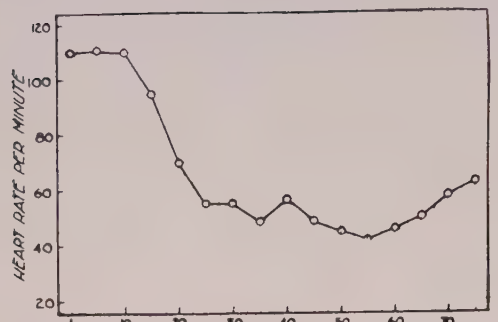

FIG. 4.

mine does not alter the cardio-inhibitory responses produced by the injection of pitressin and angiotonin. This would indicate that, as in the anesthetized animals, Dibenamine does not impair the vasoconstrictor actions of these compounds.

Summary and Conclusions. The actions of Dibenamine on the cardiovascular adjustments caused by epinephrine, acetylcholine, pitressin, and angiotonin have been studied in unanesthetized dogs. Dibenamine produced an increase in heart rate which persisted for one to 3 hours.

A test dose of epinephrine which regularly caused reflex cardiac slowing in normal unanesthetized dogs produced severe cardiac acceleration after administration of Dibenamine. This acceleration is attributable to the direct stimulatory action of epinephrine on the sino-auricular node and to reflex acceleration from a fall in blood pressure.

The compensatory cardiac acceleration produced by injection of a test dose of acetylcholine was undiminished and prolonged in unanesthetized animals under the influence of Dibenamine. This result is compatible with the interpretation that the vasoconstrictor mechanisms are impaired while the cardioaccelerator mechanisms are relatively intact.

The severe cardiac inhibition produced by pitressin and angiotonin in unanesthetized dogs, which is attributable to reflexes elicited by the rise in arterial blood pressure subsequent to vasoconstriction, was not prevented by Dibenamine.

The results of these experiments on unanesthetized dogs are in accord with the interpretations of Nickerson and Goodman[1] concerning the sites of action of Dibenamine.

16051

Diabetogenic Effect of Two Synthetic Estrogens in Force-Fed, Alloxan-Diabetic Rats.

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Ingle[1] has reported on the diabetogenic effect of diethylstilbestrol, dihydrostilbestrol, estradiol and equilin in the force-fed, partially depancreatized rat. More recently it was shown by Ingle, Nezamis and Prestrud[2] that diethylstilbestrol will intensify the glycosuria of rats having alloxan diabetes when the food intake is kept constant by forced feeding. The present study was a partial test of the hypothesis that compounds which are estrogenic are also diabetogenic in the rat. Substance I (2,4-di[p-hydroxyphenyl]-3-ethyl hexane) was described by Blanchard, Stuart and Tallman[3] and Substance II (1,2-dimethyl-2-carboxy-7-methoxy - 1,2,3,4,9,10-hexahydrophenathrene) was described by Hogg.[4]

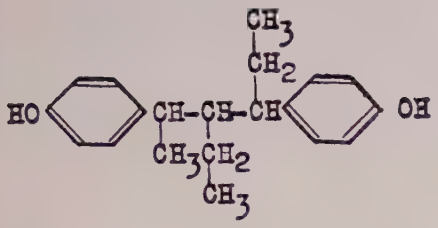
Each of these two compounds belongs to a chemically different series of synthetic estrogens than any of the substances which have previously been examined for diabetogenic

1 Ingle, D. J., *Endocrinology*, 1941, **29**, 838.

2 Ingle, D. J., Nezamis, J. E., and Prestrud, M. C., *Endocrinology*, 1947, **41**, 207.

3 Blanchard, E. W., Stuart, A. H., and Tallman, R. C., *Endocrinology*, 1943, **32**, 307.

4 Hogg, J. A., *J. Am. Chem. Soc.*, in press.



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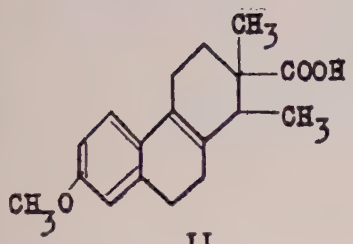

II

activity. Each compound was found to intensify the glycosuria of the alloxan-diabetic rat.

Methods. Male rats of the Sprague-Dawley strain were maintained on Purina Dog Chow until they reached a weight of 310 g. They were then fed a medium carbohydrate diet made according to Table I. During the administration of alloxan all of the rats ate the diet *ad libitum*. Alloxan was injected subcutaneously in doses of 25 mg every other day until glycosuria appeared. After a diabetic state was established the animals were placed in metabolism cages and were force-fed by stomach tube each morning (8:30 to 9:15 a. m.) and afternoon (4:15 to 5:00 p. m.). The techniques and the diet were modifications of those described by Reinecke, Ball and Samuels.[5] During the period of adaptation to

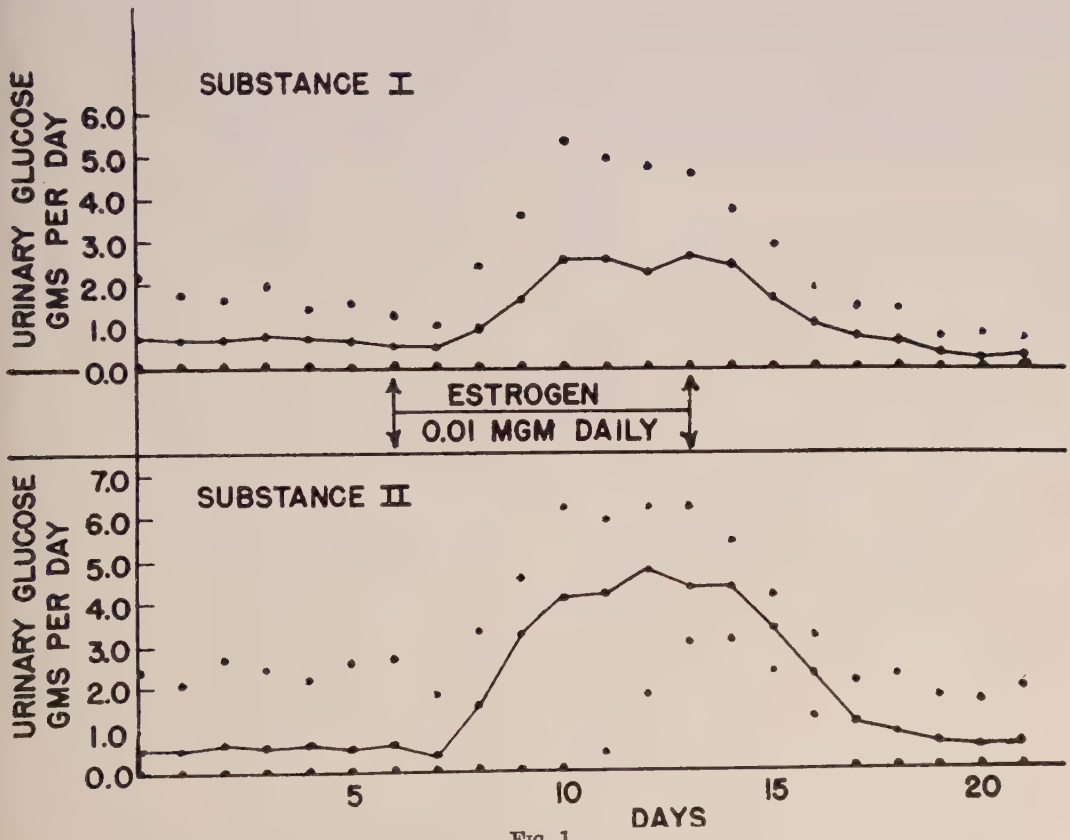

FIG. 1.

The effect of two synthetic estrogens upon the level of urinary glucose in alloxan-diabetic rats. Averages (solid line) and range of individual values. Six rats were tested in each of the two experiments.

TABLE I.
Composition of Fluid Diet.

Constituent	
Cellu flour (Chicago Dietetic Supply)	120 g
Osborne and Mendel salt mixture	40
Dried yeast (Pabst)	100
Wheat germ oil	10
Cod liver oil	10
Mazola oil	200
Vit. K (2-methyl-1-4-naphthaquinone)	100 mg
Casein (Labco)	160
Starch	200
Dextrin	190
Sucrose	200
Egg albumin (Merck)	
Water to make total volume of	2000 cc

forced feeding, the amount of diet was increased gradually to prevent the development of "food-shock." The animals were brought to a full feeding of 26 cc of diet per rat per day on the fifth day.

The animals were maintained in an air-conditioned room in which the temperature was maintained at 74-78°F and the humidity at 30-35% of saturation. Twenty-four-hour samples of urine were collected at the same hour (8:00 to 8:30 a. m.) and were preserved with thymol. Urine glucose was determined by the method of Benedict.[6]

The estrogens were made up in vegetable oil solution and were each given by subcutaneous injection once daily in a volume of 0.1 cc. Substance I (Schieffelin) was purchased on the market. Substance II was prepared by one of us (JAH) by laboratory synthesis.

Experiments and Results. Twelve rats having mild alloxan diabetes were used in these experiments. The animals were observed for a period of 3 to 4 weeks before the tests of the estrogens were started. In experiment 1, 6 rats were injected with 0.01 mg of Substance I per day for 7 days. Two of the rats were free from glycosuria during the control period. Five of the 6 rats responded to the injections of Substance I by an increase in the level of urinary glucose. When the injections were stopped the level of glycosuria decreased. The sixth rat did not excrete glucose during any phase of the experiment.

In Experiment 2, 6 rats were injected with 0.01 mg of Substance II per day for 7 days. Three of the rats were free from glycosuria during the control period, but all of the animals showed a significant increase in urinary glucose during the injection period. When the injections were stopped the glycosuria decreased in all of the animals and disappeared entirely in 4 of the 6 animals.

The data on urinary glucose are summarized in Fig. 1.

Discussion. The results of these experiments support our tentative hypothesis that compounds which are estrogenic also have the property of intensifying the glycosuria of mildly diabetic, force-fed rats. Other hormonal substances, especially those of the adrenal cortex and the anterior pituitary, are diabetogenic in the rat although they lack estrogenic activity. Of the polycyclic compounds which have been tested, only those having estrogenic activity or those having adrenal cortical activity have been found to be diabetogenic. The mechanism of diabetogenic activity in these compounds and its relationship to the role of the hormones in body economy are unknown.

These data are relative to the conclusion of Janes and Dawson[7] that estrogens are not diabetogenic in the rat and support the findings of Ingle, Nezamis and Prestrud[2] who found that diethylstilbestrol does intensify the glycosuria of the alloxan-diabetic rat just as in the partially depancreatized rat (Ingle[1]). There are two essential conditions for the exacerbation of diabetes by estrogens in the rat. First, the test animal should be mildly diabetic rather than severely diabetic. Second, the food intake must be sustained by forced feeding, otherwise the diabetogenic effect of the estrogen may be partially or completely masked by the concomitant decrease in voluntary food intake. These essential conditions were not observed in the study by Janes and Dawson.[7]

Summary. Male rats having mild alloxan

[5] Reinecke, R. M., Ball, H. A., and Samuels, L. T., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 44.

[6] Benedict, S. R., *J. A. M. A.*, 1911, **57**, 1193.

[7] Janes, R. G., and Dawson, H., *Endocrinology*, 1946, **38**, 10.

diabetes were used as test animals in this study. They were force-fed a medium carbohydrate diet. Two synthetic estrogens were tested, each of which represents a chemically different series of compounds than have previously been tested for diabetogenicity. Substance I was 2,4-di(p-hydroxyphenyl)-3-ethyl hexane, and Substance II was 1,2-dimethyl-2-carboxy-7-methoxy - 1,2,3,4,9,10-hexahydrophenathrene. Substance I caused exacerbation of the diabetes in 5 out of 6 test animals. The sixth rat did not have a spontaneous glycosuria and failed to respond to the estrogen. Substance II intensified the glycosuria in all 6 test animals and appeared to be the more potent of the two compounds. When the injections were stopped the glycosuria decreased to its pre-injection level or below.

16052

BAL Inhibition of Mercurial Diuresis in Congestive Heart Failure.*

Ralph M. Sussman and Jerome A. Schack. (Introduced by E. H. Fishberg.)

From the Medical Service of Beth Israel Hospital, New York City.

This study was undertaken to evaluate the effect of 2,3 dimercaptopropanol[1] (dithiopropanol),—commonly known as British Antilewisite or BAL—upon the diuresis induced by the organic mercurials with the view of gaining further information regarding the mechanism of mercurial diuresis, as well as controlling some of the untoward sensitivity reactions[2,3] encountered when these substances are employed. 1 - (methoxy - oxymercuri - propyl) - 3 - succinylurea—generally known as Mercuhydrin—which contains 39 mg of mercury in organic combination with 48 mg of theophyllin per cc of aqueous solution, was the sole mercurial studied. The preparation known as BAL in oil (10% 2,3 dimercaptopropanol in benzyl benzoate and peanut oil) was used.

The subjects selected were cardiac patients in chronic congestive failure who were known to respond to organic mercurial injection with diuresis and weight loss. The experience with 7 patients comprises this report. Five patients had hypertensive arteriosclerotic heart disease; one was a rheumatic cardiac and the other suffered from heart failure of undetermined etiology. Regular sinus rhythm was present in 6 of 7 of these subjects at the time of the study.

So far as feasible, the patients were maintained on the usual therapeutic cardiac regimen consisting of incomplete bed rest, salt-poor diet, containing less than 2 g of NaCl in 24 hours, no fluid restriction, and in most instances the administration of some digitalis preparation.

When it appeared that they had attained a relatively constant weight under such management, BAL in oil was introduced for a period of 24 to 36 hours. It was administered intramuscularly every 6 hours in doses of 2.5 mg per kilo of body weight. After receiving BAL in oil for 24 hours, Mercuhydrin was injected intramuscularly in 2 cc doses. The BAL in oil was then continued for a period of 12 hours.

Table I contrasts the weight changes occurring in patients receiving BAL in oil for about 24 hours, prior to the administration of Mercuhydrin with those who received the mercurial only.

The individaul weight loss 48 hours after

* Work done under a grant from the Joseph and Helen Yeamans Levy Foundation in memory of Miriam Levy Finn.

1 Peters, R. A., Stocken, L. A., and Thompson, R. H. S., *Nature*, 1945, **156**, 616.

2 Brown, G., Friedfeld, L., Kissin, M., Modell, W., and Sussman, Ralph M., *J. Am. Med. Assn.*, 1942, **119**, 1004.

3 Long, W. K., and Faran, A., *Science*, 1946, **104**, 220.

TABLE I.
Weight Change.

Subject	BAL with Mercurial		Mercurial	
	24 hr	48 hr	24 hr	48 hr
J.D.	— ¾	+ ¾	—3¼	—5¼
S.S.	+ ½	+1½	—4¼	—3¾
H.G.	0	+1	—4	—3½
E.G.	— ½	— ¼	—3½	
F.S.	— ¾	— ½	—2¾	—3¾
G.N.	0	+1	—3¾	—2¾
N.R.	— ¼	+1	—1¾	—3¼
Avg	— ¼	+ ⅗	—3⅓	—3⅔

the administration of the mercurial, alone, was somewhere between 2¾ and 5¼ lb, whereas, patients receiving BAL in oil with the mercurial, in most instances gained weight, only 2 having lost ½ lb or less.

Some observations made by varying the period of BAL administration preliminary to mercurial injection indicate that at least 4 doses of 2.5 mg per kilo body weight injected at intervals of 6 hours must be given prior to the introduction of the 2 cc of the mercurial in order to inhibit diuresis completely. It is also evident from this investigation that within 48 hours after the last injection of BAL in oil, diuresis can again be anticipated from another adequate injection of mercurial. Patients who were observed for several days following the cessation of BAL in oil injections displayed no evidence of diuresis from the mercurial administered simultaneously with the dithiopropanol. It may, therefore, be concluded that BAL in oil completely annuls the diuretic action of the mercury and does not merely delay it.

Notes made on the side-reactions experienced with BAL in oil coincide with earlier pharmacologic observations. Thus, transient rise in blood pressure, local reaction, nausea, vomiting, warmth, paraesthesias and chill were all observed.[4]

In 2 hypertensive patients, alarming elevation of systolic and diastolic blood pressures coincided with the administration of BAL in oil and persisted for at least 4 hours. In one patient the symptoms were interpreted as being due to "hypertensive encephalopathy".[5]

Considerable local pain was experienced at the site of injection and the administration of 0.5 cc of 1% procaine in the syringe with each injection relieved one patient considerably without in any way diminishing the anti-diuretic effect of the dithiopropanol.

No cases of organic mercurial sensitivity were available at the time of study, so that no remarks regarding the response of such patients to BAL can be made.

Unless differences in species reaction occur, it is thought that the mechanism of BAL inhibition of mercury diuresis results from the formation of a stable mercury-thiol complex,[6,7] binding free mercury, and that BAL is not, itself, an anti-diuretic substance.

These findings further substantiate the notion that the diuresis from organic mercurials is due to the systemic effect of free mercury.

Since this study was completed, an important communication from Handley and La Forge has appeared and has established that BAL injected intravenously into dogs inhibits mercurial diuresis in that animal.[8] Their findings in the normal dog are in complete accord with our observations in humans suffering from heart failure.

Conclusions. 1. BAL in oil completely annuls the diuretic effect of the organic mercurial known as Mercuhydrin.

2. A period of priming with BAL in oil was necessary to inhibit the diuresis completely.

3. Procaine may be added to BAL in oil without in any way affecting the anti-diuretic effect.

4. BAL in oil may intensify pre-existing hypertension in the presence of heart failure.

4 Modell, Walter, Gold, Harry, and Cattell, McKeen, *J. Clin. Invest.*, 1946, **25**, 480.

5 Fishberg, A. M., *Hypertension and Nephritis*, Chapter X, Lea & Febiger, 4th edition, 282.

6 Barron, E. S. G., and Kalnitsky, G. (quoted by Longcope, W. T., and Luetscher, J. A.), *J. Clin. Invest.*, 1946, **25**, 557.

7 Gilman, Alfred, Allen, Roberta P., Philips, Frederick S., and St. John, Ellen, *J. Clin. Invest.*, 1946, **25**, 549.

8 Handley, Carroll A., and La Forge, Marguerite, Proc. Soc. Exp. Biol. and Med., 1947, **65**, 74.

16053 P

Isolation of Western Equine Encephalomyelitis Virus from Tropical Fowl Mites, *Liponyssus bursa* (Berlese).*

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From the Department of Bacteriology and Immunology and the Virus Research Laboratory, Southwestern Medical College, Dallas, Texas.

At the present time the exact mode of transmission of western equine encephalomyelitis is far from established, and the mechanism by which the causative agent persists from year to year in a given area has not been fully explained. Numerous attempts have been made to determine reservoir hosts and vectors of the disease. Hammon and his associates[1] demonstrated that a large per cent of both domestic and wild birds possess neutralizing antibodies for this virus suggesting that the reservoir host might be one closely associated in some manner with domestic fowl. It seemed likely, as pointed out by Smith and her associates,[2] that some blood-sucking vector which does not necessarily bite man was transmitting the disease to fowl. These investigators recently reported the isolation of the St. Louis encephalitis virus from naturally infected chicken mites, *Dermanyssus gallinae* (DeGeer), in the St. Louis area where the disease is endemic. Later, they reported several additional isolations from chicken mites, and demonstrated transovarian transmission of the virus from naturally infected female mites.[3] Shortly thereafter, Sulkin[4] reported the isolation of the Western equine virus from naturally infected chicken mites in the Dallas area during the epidemic year of 1944. During the following non-epidemic year additional attempts to demonstrate this virus in chicken mites and fowl ticks collected in this identical locality and other areas in Texas, were unsuccessful. Numerous attempts by Hammon and his associates[5] to demonstrate virus in this ectoparasite have been negative. Since the possible role of the chicken mite in the transmission and effective perpetuation of the virus encephalitides is not yet clarified, it was considered desirable to continue the search for a reservoir among other avian blood-sucking ectoparasites.

Recently Reeves and his collaborators[6] reported recovery of the Western equine virus from wild bird mites, *Liponyssus sylviarum* (Canestrini and Fanzago), in Kern County, California. The present report concerns an additional isolation of the Western equine virus from similar mites collected in Dallas County, Texas from a nest containing two live nestling English sparrows, *Passer domesticus* (Linn.).† The nest was brought into the laboratory on July 9, 1947. A layer of white absorbent cotton was placed over the top, and the entire nest was then placed in a covered glass container. On the following day, mites identified as *Liponyssus bursa* (Berlese),† were collected from the cotton and the underside of the lid, and were tested

* This investigation was aided by a grant from the Rose Lampert Graff Foundation, Los Angeles, California.

1 Hammon, W. McD., Lundy, H. W., Gray, J. A., Evans, F. C., Bang, F., and Izumi, E. M., *J. Immunol.*, 1942, **44**, 75; Hammon, W. McD., Reeves, W. C., and Irons, J. V., *Texas Rep. Biol. and Med.*, 1944, **2**, 366.

2 Smith, M. G., Blattner, R. J., and Heys, F. M., *Science*, 1944, **100**, 362.

3 Smith, M. G., Blattner, R. J., and Heys, F. M., Proc. Soc. Exp. Biol. and Med., 1945 **59**, 136; *J. Exp. Med.*, 1946, **84**, 1.

4 Sulkin, S. E., *Science*, 1945, **101** 381.

5 Hammon, W. McD., and Reeves, W. C., *Am. J. Pub. Health*, 1945, **35**, 994.

6 Reeves, W. C., Hammon, W. McD., Furman, D. P., McClure, H. E., and Brookman, B., *Science*, 1947, **105**, 411.

† The authors are indebted to Dr. E. P. Cheatum, Department of Biology, Southern Methodist University, for confirming the identifications, and to George L. Carpenter, Typhus Control Supervisor, United States Public Health Service, for collecting specimens for study.

in 3 pools for the presence of a neurotropic virus. The three pools, each containing approximately 300 mites (adults and nymphs), were aspirated into small vials as described elsewhere[7] and allowed to remain overnight. Each pool was ground in a mortar with 2.0 cc of nutrient broth, and then centrifuged at 5000 r.p.m. for 15 minutes in an angle centrifuge. Approximately 0.3 cc of the supernatant fluid, which was cultured aerobically and anaerobically, was injected intraperitoneally into each of 5 Swiss mice. Pool No. 1 was inoculated into 8-day-old mice and pools No. 2 and No. 3 into 12-day-old mice. The supernatant fluids did not contain enough bacteria to affect the animals. An agent later identified as western equine encephalomyelitis virus was recovered from pool No. 1 and after 4 serial passages in Swiss mice, filtration experiments (Berkefeld N filter) were conducted with the pooled brains of mice showing neurological symptoms. Identification of a filterable agent of the sixth passage as the Western type of equine encephalomyelitis virus was made by neutralization tests with serum of rabbits immunized to known strains of Western and Eastern equine virus and St. Louis encephalitis virus. The virus recovered from the sparrow mites was neutralized by the Western equine immune serum but was not affected by the serum of rabbits immunized to the Eastern strain of equine virus or the virus of St. Louis encephalitis. With each passage, before and after the filtration experiment, aerobic and anaerobic cultures were made and brain tissue from animals showing central nervous system symptoms were examined for histopathological evidence of encephalitis. Microscopic sections of a mouse and a guinea pig brain from the second intracerebral passage showed histopathological evidence characteristic of encephalitis. Pools No. 2 and No. 3 of mites collected from the same source but injected into older mice yielded negative results. All attempts to isolate a filterable virus from 11 additional pools of specimens of wild bird mites yielded negative results. Additional attempts to demonstrate virus in chicken mites were likewise negative. Studies are still in progress to determine whether or not hereditary transmission of the Western equine virus in the chicken mite can be effected under experimental conditions. Similar studies will also be made with the wild bird mite.

Thus, in addition to the well documented evidence incriminating the mosquito in the transmission of western equine encephalomyelitis,[5,8] it is becoming increasingly apparent that other blood-sucking arthropods may be concerned in the transmission of the virus of equine encephalomyelitis.

Summary. The Western type of equine encephalomyelitis virus has been isolated from wild bird mites, *Liponyssus bursa* (Berlese), collected in the Dallas County, Texas area. At the present time the epidemiological role played by the wild bird mite and the chicken mite, *Dermanyssus gallinae* (DeGeer), from which the Western equine virus had been previously isolated, is unknown. The purpose of this report is to emphasize the importance of virus isolations from similar bird mites in two widely separated areas.

ADDENDUM: Two months after the Western equine virus was isolated from the tropical bird mites collected in Dallas County, the Eastern equine virus was recovered from a horse brain submitted to us from Jefferson County, Texas, where a widespread epizootic was occurring. This would indicate that both the Western and Eastern equine types of encephalomyelitis were active in Texas at about the same time.

7 Wisseman, C. L., Jr., and Sulkin, S. E., *Am. J. Trop. Med.*, 1947, **27**, 463.

8 Hammon, W. McD., Reeves, W. C., Brookman, B., and Izumi, E. M., *J. Inf. Dis.*, 1942, **70**, 263.

16054

Indoleacetic Acid and Growth of Bacteria with Varying Requirements for Nicotinic Acid and Tryptophane.

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An antagonism between indole-3-acetic acid, and nicotinic acid and/or tryptophane, was suggested by the data of Kodicek, Carpenter, and Harris[1] as a possible explanation for the role of corn in the etiology of pellagra. It was thought of interest to study the relationship between these compounds in certain bacteria whose nutritional requirements are relatively well known, and which can be grown in chemically defined media.

The following organisms were selected (numbers in parentheses refer to American Type Culture Collection listings): Requiring both nicotinic acid and tryptophane: *Lactobacillus casei* (7469), *Streptococcus faecalis* R (8043), and *Lactobacillus arabinosus* 17-5 (8014). Shankman *et al.*, state[2] that the nicotinic acid requirement of the latter organism is abolished upon long incubation. Requiring nicotinic acid alone: *Staphylococcus aureus*; requiring neither: *Escherichia coli* and *Aerobacter aerogenes*.

After this work was initiated, Dubos[3] reported that indoleacetic acid in concentrations of 0.01 to 0.10 mg/ml inhibited *Mycobacterium tuberculosis* (Human strain), certain streptococci, and *Shigella paradysenteria* (Sonne) grown in enzymatic casein hydrolyzate-yeast extract medium at pH 6.0. This inhibition was reported to be relieved by concentrations of added tryptophane ten times that of the inhibitor.

Experimental. The lactic acid bacteria and *S. aureus* were grown on either the amino acid medium of Stokes *et al.*,[4] with sodium citrate substituted for sodium acetate, or, in most cases, on the same medium with acid-hydrolyzed casein substituted for the amino acid medium. These variations produced no significant alteration in results. *E. coli* and *Aerobacter aerogenes* were grown on MacLeod's[5] medium of glucose, ammonium sulfate, asparagine, and salts. Following the appearance of Dubos' report, a non-pathogenic strain of Mycobacterium tuberculosis (American Type Culture Collection No. 607) was tested, using Long's synthetic medium.

In all cases, indoleacetic acid, nicotinic acid, and tryptophane were adjusted to the pH of the respective media, and added as required. The medium was autoclaved following these additions. Except in the case of *M. tuberculosis*, where a small fragment of mycelium was employed for inoculation, the inoculum consisted of one drop of a saline suspension of organisms which had been washed with saline and then suspended in 2.5 times the original culture volume.

For lactic acid bacteria, growth was measured after 48 to 72 hours incubation at 37°C by titration with 0.1 N NaOH to pH 6.8 on a Berkman pH meter. Growth of *M. tuberculosis* was measured, after either 3 or 6 days incubation, by filtering, washing, drying, and weighing the mycelium. Growth of the other organisms was measured as turbidity (expressed as optical density) in the Evelyn Photoelectric Colorimeter after 24 to 48 hours incubation. In none of these cases did variations in incubation time significantly alter results.

Results. The results of these experiments are indicated in Table I. Each indicated value

1 Kodicek, E., Carpenter, K. J., and Harris, L. J., *The Lancet*, 1946, **251**, 491.

2 Shankman, S., Camien, M. N., Block, H., Merrifield, R. B., and Dunn, M. S., *J. Biol. Chem.*, 1947, **168**, 23.

3 Dubos, R. J., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 317.

4 Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, 1945, **160**, 35.

5 MacLeod, C. M., *J. Exp. Med.*, 1940, **72**, 217.

TABLE I.
Effect of Indoleacetic Acid, Nicotinic Acid, and Tryptophane upon Certain Bacteria.

Organism	Exp. No.	IAc (mg/ml)	Growth with indicated concentrations of NA and T (mg/ml) T=0.3 NA=0.01	T=0.3 NA=10	T=30 NA=0.01	T=30 NA=10
Streptococcus fæcalis R	1	0	3.8	5.5	3.8	5.4
Growth measured as ml of		0.1	3.4	5.3	3.4	5.2
0.1 N NaOH		1.0	2.5	—	2.5	3.8
	2	0	4.8	5.2	3.3	5.5
		0.1	4.4	5.0	3.4	4.9
		1.0	3.6	—	—	4.7
	3	0	2.7	3.0	3.5	7.8
		0.1	2.5	2.8	3.3	5.7
		1.0	2.4	3.1	3.1	5.6
	4	0	2.4	2.9	3.5	7.0
		0.1	1.7	2.6	3.4	6.4
		1.0	2.0	2.0	2.7	4.9
Lactobacillus arabinosus 17-5 (8014)	1	0	2.2	2.7	6.0	8.2
Growth measured as ml of		0.1	2.6	3.2	5.8	8.4
0.1 N NaOH		1.0	2.7	5.1	4.2	3.1
	2	0	2.0	2.9	3.2	9.9
		0.1	1.8	3.0	3.2	10.1
		1.0	2.4	2.8	1.4	4.4
Lactobacillus casei (7469)	1	0	2.9	2.9	6.6	—
Growth measured as ml of		0.1	3.1	3.3	6.3	7.8
0.1 N NaOH		1.0	—	4.7	4.0	6.1
	2	0	2.6	3.8	5.1	7.3
		0.1	3.1	3.8	3.0	7.5
		1.0	2.8	—	1.0	3.5
Staphylococcus aureus	1	0	0.31	0.30	—	0.31
Growth measured as optical density		0.1	0.35	0.39	—	0.41
		1.0	0.31	—	—	0.33
	2	0	0.28	0.36	0.33	0.42
		0.1	0.34	0.37	0.32	0.40
		1.0	0.30	0.39	0.32	0.43
Escherichia coli	1	0	0.18	0.17	0.15	0.17
Growth measured as optical density		0.1	0.18	0.17	0.17	0.17
		1.0	0.13	0.10	0.11	0.12
Aerobacter aerogenes	1	0	0.52	0.52	—	0.51
Growth measured as optical density		0.1	0.40	0.53	—	0.50
		1.0	0.13	0.14	—	0.14
	2	0	0.18	0.16	—	0.23
		0.1	0.17	0.14	—	0.17
		1.0	0.10	0.11	—	0.10

IAc = Indole-3-acetic acid.
T = *l*-tryptophane.
NA = Nicotinic acid.

for growth is based upon duplicate measurements.

In addition to the data given in Table I, *L. arabinosus* was grown on the basal medium supplemented with 1.0 mg/ml of nicotinic acid, and 1.0 mg/ml of indoleacetic acid. Varying amounts of tryptophane, from 1 γ to 1 mg/ml, were added, with no relief of inhibition.

In the case of *M. tuberculosis*, the results shown in Table II were obtained after 6 days incubation. Similar results, at lower levels of growth, obtained after 3 days incubation.

Discussion. From the results of these experiments, it can be seen that although indoleacetic acid may act as an inhibitor at relatively high concentrations (1 mg/ml), and, sometimes, to a smaller extent, at concentrations of 0.1 mg/ml, this inhibition appears to bear

TABLE II.
Effect of Indoleacetic Acid on Growth of *M. tuberculosis.*

IAc mg/ml	T mg/ml	NA mg/ml	Growth mg dried mycelium
0	0	0	.167
0	0	2	.144
0	2	0	.157
.2	0	0	.151
.2	0	2	.167
.2	2	0	.137

no relationship to the nicotinic acid or tryptophane requirements of the organism, and is not relieved, in the organisms studied, by either of the latter two substances. In fact, the organism most strongly inhibited by indoleacetic acid, *Aerobacter aerogenes,* requires neither tryptophane nor nicotinic acid for growth. It is interesting to note that *E. coli,* for which stimulation by indoleacetic acid at levels up to one γ/ml in a medium containing 0.1 mg/ml of tryptophane has been reported,[6] was unaffected by the lower concentration of indoleacetic acid used in this study, and was inhibited by the higher concentration. *S. aureus* showed no inhibition at the highest concentration of indoleacetic acid used.

In certain cases, particularly *L. arabinosus* and *L. casei,* a stimulatory effect at 0.1 mg/ml, and sometimes at 1 mg/ml, levels of indoleacetic acid was observed when the concentration of tryptophane was limiting; this stimulation disappeared at adequate levels of tryptophane regardless of nicotinic acid content. The latter organisms are not specific in their response to tryptophane,[7,8] since indole and anthranilic acid have been reported to have tryptophane activity. However, indoleacetic acid was stated to be inactive. It should be noted, however, that the latter studies[7,8] measured the response of these organisms to tryptophane derivatives in media completely devoid of tryptophane; it is possible that indoleacetic acid may stimulate only in the presence of small amounts of the amino acid.

6 Ball, E., *J. Bact.,* 1938, **36**, 559.

7 Snell, E. E., *Arch. Biochem.,* 1943, **2**, 389.

8 Greene, R. D., and Black, A., *J. Biol. Chem.,* 1944, **155**, 1.

It is realized that Dubos obtained relief of inhibition at concentrations of added tryptophane 10 times that of inhibitor, and that this ratio was not used in the present experiments. Further, Dubos used a basal medium already containing tryptophane in the enzymatic casein hydrolyzate and yeast extract. Because of these facts, our results and those of Dubos are not strictly comparable. Since our inhibition was generally obtained with one mg/ml of indoleacetic acid, using Dubos' ratio would not only have led to difficulties due to solubility, but would have produced such a high concentration of tryptophane as to make interpretation of results difficult. If a true metabolite-antagonist relationship exists between indoleacetic acid and nicotinic acid-tryptophane, it is a relatively unusual phenomenon that far greater concentrations of metabolite than of the antagonist are required to relieve inhibition. This fact, as well as the lack of relationship between nicotinic acid and tryptophane requirements of organisms and their susceptibility to indoleacetic acid inhibition, tend to detract from the probability of a true metabolite-antagonist relationship. A recent report by Krehl, Carvalho, and Cowgill,[9] failing to confirm the results of Kodicek *et al.*,[1] should be noted. It would appear that a metabolite-antagonist relationship between indoleacetic acid and nicotinic acid or tryptophane is not an invariably occurring biological phenomenon, nor is there any evidence indicating the mechanism of this reaction where it does occur.

Summary and Conclusions. 1. A number of organisms whose tryptophane and nicotinic acid requirements are known were tested for possible inhibition by indoleacetic acid and reversal by nicotinic acid and tryptophane in chemically defined media.

2. *Escherichia coli, Lactobacillus arabinosus* 17-5, *L. casei, Streptococcus faecalis R,* and *Aerobacter aerogenes* were inhibited by concentrations of indoleacetic acid of one mg/ml Occasionally slight inhibitions were noted at 0.1 mg/ml levels.

9 Krehl, W. A., Carvalho, A., and Cowgill, G. R., *Fed. Proc.,* 1947, **6**, 413.

3. No significant relief of inhibition was obtained with either nicotinic acid or tryptophane.

4. *Staphylococcus aureus* and *Mycobacterium tuberculosis* (607) were not inhibited by indoleacetic acid at the concentrations studied.

5. Fairly consistent stimulation at 0.1 mg/ml levels of indoleacetic acid were obtained with *L. arabinosus* and *L. casei* in the presence of limiting concentrations of tryptophane.

6. The significance of these results in relation to possible metabolite-antagonist relationship between indoleacetic acid and tryptophane or nicotinic acid is discussed.

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16055

Treatment of Experimental Intestinal Trichinosis with 1-Diethylcarbamyl-4-Methylpiperazine Hydrochloride (Hetrazan*).

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The drug 1-Diethylcarbamyl-4-methylpiperazine Hydrochloride (Hetrazan), when administered orally, causes the rapid disappearance of the microfilariae of *Wuchereria bancrofti* from the blood of infected individuals.[1] The marked effect of this drug on the circulating forms of *W. bancrofti* suggested its use in other parasitic infections in which treatment should be directed against such migrating forms. In view of the need for a drug in trichinosis which would destroy the migrating larvae as well as the intestinal forms, Hetrazan was tested on white rats infected with *Trichinella spiralis*. The effect of this drug on the intestinal trichinae is reported below.

Methods and Results. White rats weighing from 150 to 175 g were fed by stomach tube with 1,000 to 1,300 infective trichinae larvae. The larvae were obtained from rat muscle digested in a pepsin-hydrochloric acid mixture. Twenty-four hours after feeding the animals were started on Hetrazan. The drug was given on the basis of 200 mg per kg weight 3 times daily by stomach tube, for periods of 5 to 10 days. For the recovery of adult trichinae the treated animals and suitable controls were killed 24 hours after the last dose of the drug was administered. The small and large intestine were removed, slit open and cut into small pieces about 1 cm long. The sliced intestine was put in a Baermann apparatus with normal salt solution heated to 37°C. The adult worms settled rapidly to the tip of the funnel and were removed one hour after the apparatus was set up. The number of worms recovered from the intestines was determined directly by counting.

For the recovery of the muscle stages the rats were killed on or about the 30th day after feeding infective larvae. The rat muscles were ground and digested in a pepsin-hydrochloric acid mixture. The larvae were recovered from the mixture and their number estimated by counting aliquot portions of the total suspension.

The average number of adult worms recovered from the rats treated with Hetrazan was considerably less than the number recovered from the untreated animals (Table I). Thus the average number of worms

* The drug was supplied by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y.

1 Santiago, D., Oliver-González, J., and Hewitt, R. I., in press.

TABLE I.

Effect of Hetrazan on Experimental Trichinosis as Determined by the Number of Adult and Larval Trichinæ Recovered from Rats. Six Rats Used in Each Experiment.

No. of infective larvæ fed per rat	Amt of drug given orally per rat (mg per kilo wt T.I.D.)	Length of treatment, days	Days after feeding infective larvæ when killed					
			5		10		30	
			Avg No. of worms recovered from intestine	Avg No. of larvæ recovered from muscle	Avg No. of worms recovered from intestine	Avg No. of larvæ recovered from muscle	Avg No. of worms recovered from intestine	Avg No. of larvæ recovered from muscle
1,000	200	5	91	0				
"	0		481	0				
"	200	10			312	0		
"	0				61	0		
1,300	200	5					0	320
"	0						0	31,200
"	200	10					0	112
"	0						0	26,100
"	200	20					0	2

recovered in 6 rats treated with Hetrazan for 5 days was 91, as compared with 481 which was the average number of worms recovered in the untreated animals.

Similarly the average numbers of larvae recovered from the muscles of the treated rats were much less than those recovered from the untreated animals.

Discussion and Summary. According to the results presented above, Hetrazan reduces the number of adult *Trichinella spiralis* when the drug is administered orally to rats infected with this parasite. That the number of adult worms is reduced is also shown by the fact that the larvae recovered from the musculature of the rats, treated early during infection, are less in number than those recovered from the untreated rats.

Hetrazan, when administered orally to man is relatively non-toxic.[1] The amount of drug required to kill the microfilariae of *W. bancrofti* is well under the lethal dose for rats (one mg per kg in man as compared with an LD_{50} of 285 mg per kg in rats). Hewitt *et al.*[2] have reported that the amount of drug necessary to kill the cotton rat filarid is from 10 to 25 mg per kg which has to be given constantly for a period of 2 to 4 weeks. However, the amount of drug necessary to act on the filarid worm of man is considerably less (one mg per kg 3 times daily for 5 days). Although the same species of *Trichinella* is involved in infection of man and rat it may be that, as in the case of the filarial infections, the amount of the drug necessary to overcome the infection in man is less than the amount needed for the treatment of rats.

Hetrazan acts on other intestinal nematodes particularly on *Ascaris* of man and dog.[3] It is suggestive that this drug may be useful mixed with the animal feed in order to treat infections with *Ascaris* and intestinal trichinae.

Work is in progress to determine the effect of Hetrazan on the migratory and muscle stages of *T. spiralis*.

[2] Hewitt, R., Kushner, S., Stewart, H., White, D. E., Wallace, W., and SubbaRow, Y., in press.

[3] Unpublished data.

16056

Plasma Levels and Urinary Excretion of Injected Myanesin in Dogs.*

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The recent publications of Berger and Bradley[1,2] describing a new synthetic curarizing agent, α-β-dihydroxy-γ-(2-methylphenoxy)- propane (myanesin), and the initial clinical report by Mallinson[3] of its use as a substitute for curare in 118 cases, are of potential interest in the field of anesthesia.

The brevity of action of myanesin aroused our interest in its physiological disposition, and it was considered desirable to study blood levels and urinary excretion. This report describes a method developed for the estimation of myanesin in plasma and in urine, and experimental data of plasma levels and urinary excretion in dogs.

Principle of the Method. The method described here is dependent upon the nitration of myanesin in aqueous solution, and the development of a strong yellow-green color when made alkaline with sodium hydroxide. The yellow-green color is stable for at least 60 minutes, and its intensity is measured with the photoelectric colorimeter. The myanesin is extracted from blood plasma, or from urine, with chloroform and the chloroform evaporated to dryness. The residue is then dissolved in water and an aliquot analyzed. By acid hydrolysis of the urine before the extraction with chloroform much larger quantities of myanesin can be recovered, indicating the presence of a conjugated derivative of myanesin in urine.

Method of Analysis. Extraction. (1) Plasma.‡ Five ml of plasma obtained from oxalated blood are placed in a small separatory funnel, and 10 ml of chloroform (technical grade) are added. After thorough shaking for 8 minutes the chloroform layer is separated from the plasma, and filtered through Whatman No. 40 filter paper. Occasionally, especially in hot weather, the plasma and the chloroform may emulsify, but rarely is it impossible to obtain 5 ml of clear chloroform filtrate. A 5 ml aliquot of the chloroform filtrate is evaporated just to dryness on a water bath heated to 90°C, being careful not to overheat the residue. Two and five-tenths ml of distilled water are added to the residue, and gently warmed to aid solution of the myanesin. After a few minutes, 2 ml of this aqueous solution are placed in a test tube 16.5 x 150 mm, to be submitted to analysis. This 2 ml aqueous fraction represents the extracted myanesin from 2 ml of plasma.

(2) Urine.‡ (a) Extraction of free myanesin. Four and five-tenths ml of urine are placed in a small separatory funnel and 1.5 ml of buffer having a pH of approximately 7.3 (prepared by adding 0.1 M citric acid to 0.2 M Na_2HPO_4 in the proportion of 2:13) are added. Twelve ml of chloroform are added, and the procedure as outlined for plasma followed, except that an aliquot of 8 ml of chloroform filtrate is evaporated to dryness, and the residue is dissolved in 3 ml of water; the 2 ml of aqueous solution of urine extract placed in the test tube for analysis represent 2 ml of urine.

(b) Extraction of free and conjugated myanesin. In a small flask 2.5 ml of urine are placed, and 2.5 ml of conc. HCl (sp. gr. 1.178-1.188) are added. The contents are then heated on a boiling water bath for 10 minutes.

* Supported by a grant from Parke, Davis & Co.

† The authors wish to acknowledge the technical assistance of Miss Millicent Svoboda.

1 Berger, F. M., and Bradley, W., *Brit. J. Pharmacol.*, 1946, **1**, 265.

2 Berger, F. M., and Bradley, W., *Lancet*, 1947, **1**, 97.

3 Mallinson, F. B., *Lancet*, 1947, **1**, 98.

‡ Reagent blanks of myanesin-free plasma and urine must be run concomitantly, since small amounts of plasma and urine constituents are extracted and nitrated.

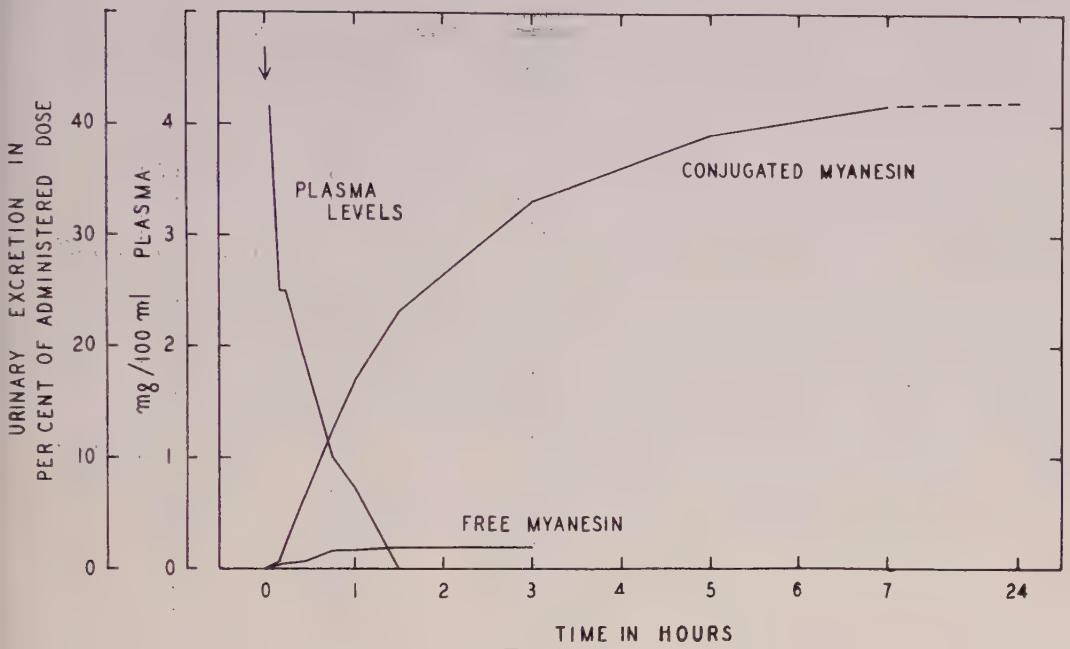

FIG. 1.
Plasma Levels and Urinary Excretion of Myanesin.
The arrow in the upper left hand corner represents the time of the intravenous injection of myanesin, 50 mg per kg. Values plotted correspond to those of Dog No. 4 in Table II.

(Autoclaving similar solutions for 2 and 4 hours at 115-120° C did not increase the recovery of myanesin.) After removal from the bath, 5 ml of water are added to the flask, and well mixed. Five ml are then withdrawn and placed in a small separatory funnel, and the procedure as outlined under plasma followed. The 2 ml of aqueous solution eventually analyzed represent 0.5 ml of urine; consequently the final concentration value must be multiplied by 4 to yield a value reading in mg per 100 ml for the 2 ml analyzed. Note: Analysis of urine for free myanesin must be accomplished within a few hours after the sample is obtained, since spontaneous hydrolysis of the conjugated fraction gradually increases the values of free myanesin.

Analysis. Two ml of the aqueous solution of the plasma or urine extract are placed in a test tube. To each tube is added 1 ml of conc. nitric acid (sp. gr. 1.42). The tubes are then placed in a boiling water bath for exactly 4 minutes. The tubes must not be inserted until the temperature of the water is at least 98°C. Less than 3 minutes in the bath gives inadequate nitration; greater than 5 minutes contributes heavily to the destruction of some of the myanesin present. At the end of 4 minutes the tubes are removed and placed immediately in a beaker of cold water. When the solutions have reached room temperature, 2 ml of 40% sodium hydroxide are slowly added to each tube, with swirling to insure complete mixture.

Ten minutes after the addition of the sodium hydroxide, the contents of each tube are transferred to a clean, dry Klett tube and read in the colorimeter. The plasma or urine reagent blanks are set to zero; readings can then be converted into concentration values directly by comparison with the standard curve.

A Klett-Summerson photoelectric colorimeter was used in our determinations, employing the No. 42 (blue) filter (400-460 millimicrons wave length transmission). Using the method outlined above, the standard curve was prepared with aqueous solutions of myanesin of known concentration, and

TABLE I.
Recovery of Myanesin Added to Plasma and to Urine.

Sample	Myanesin added (γ/ml)	Myanesin recovered (γ/ml)	% recovery
5 ml dog plasma	25	24.0	96.0
	50	48.5	97.0
	75	69.0	92.0
5 ml human plasma	10	10.0	100.0
	100	86.8	86.8
		Avg	94.4 ± 2.3%
4.5 ml dog urine*	7.5	5.1	68.0
	10	7.4	74.0
	50	42.3	84.6
	75	61.0	81.3
4.5 ml human urine	10	9.0	90.0
	25	24.0	96.0
	75	67.0	89.3
		Avg	83.3 ± 4.0%
2.5 ml dog urine†	10	12.4	124.0
	25	27.6	110.4
	50	47.8	95.6
	50	52.0	104.0
	50	38.0	76.0
	50	38.1	76.2
	50	34.5	69.0
	75	70.1	93.5
	100	95.6	95.6
		Avg	93.8 ± 5.9%

* Analyzed directly for free myanesin.
† Submitted to procedure for hydrolysis before analysis.

plotted to read in mg per 100 ml. The reading of the reagent blank was adjusted to zero on the colorimeter. A linear relationship exists between concentrations of myanesin and colorimeter readings for values from 0.5 to 10.0 mg per 100 ml (5 to 100 gamma per ml) of solution analyzed. Periodic checks on aqueous solutions of myanesin, ranging from 0.001 to 1.0% in concentration, showed that such solutions undergo no deterioration for at least 30 days, if stored in a refrigerator.

Experimental. Recovery Data. Table I shows recovery data obtained from analysis of dog and human plasma and urine to which known amounts of free myanesin have been added. Recovery from plasma yields values 94.4 ± 2.3% of the theoretical concentration values; from urine by analysis for free myanesin 83.3 ± 4.0% of the theoretical values; from urine containing free myanesin but submitted to treatment with hot hydrochloric acid 93.8 ± 5.9% of the known values. No conjugated myanesin was available to test hydrolysis and recovery by this method. Probably the increase in % recovery from urine after acid treatment was due to greater extractability. However, the method outlined gave maximal values with urine from dogs previously given myanesin.

Plasma Levels. (Table II). Four dogs after absence of dehydration was assured were given myanesin intravenously in doses of 50 mg per kg and one dog, 75 mg per kg, employing 2-3% supersaturated aqueous solutions.[1] Three were followed as to plasma levels by periodically withdrawing blood samples. Fig. 1 shows a typical plasma level curve (Dog No. 4 of Table II). The sharp decay of the curve of free myanesin correlates well with the response of the animal. Immediately following the injection of 50 mg per kg, the dogs exhibited a flaccid paralysis, affecting predominently the hind legs. No reflexes or responses to painful stimuli could be elicited. Within 2 minutes after the injection all dogs were able to stand, though unsteadiness and muscular weakness persisted for about 20 minutes. Three of the 5 dogs

TABLE II.
Plasma Levels and Urinary Secretion.

	Dog	Solution	Time in min.								
			5	10	15	25	40	60	90	180	
Mg myanesin per 100 ml plasma	1	Plasma	4.90	2.85	2.42	2.35	0.85	0.70	0.0	—	
	3	,,	2.68	2.10	—	1.22	1.50	0.88	0.0	0.0	
	4	,,	4.16	2.50	2.50	1.90	1.00	0.73	00	—	
			Time in min.				Time in min.				
			10	20	25	40	45	60	90	120	180	300
Free myanesin in % of administered dose	1	Urine	—	0.016	—	0.054	—	0.084	0.107	0.119	0.133	—
	3	,,	0.132	—	0.275	0.433	—	0.527	0.527	—	0.527	—
	4	,,	0.19	—	0.32	—	1.57	1.62	1.93	—	1.93	1.93
			Time in min.				Time in min.					
			10	25	40	60	90	180	240	300	420	24 hrs
Combined myanesin in % of administered dose	2†	Urine	—	—	—	—	—	—	33.7	—	38.1	40.2
	3	,,	1.51	5.31	10.41	14.92	23.02	27.06	—	30.98	31.18	31.18
	4	,,	0.62	6.2	12.4	16.8	22.4	32.6	—	39.0	41.9	42.0
	5*†	,,	—	—	—	—	—	28.2	—	—	—	34.0

* Given 75 mg per kg myanesin.
† Foley bag catheter not employed here.

given myanesin vomited within 2 minutes after the injection. No free myanesin could be detected in the 90-minute plasma sample of any of the dogs. Conjugated myanesin, though probably present in plasma, could not be detected quantitatively by the above method of acid hydrolysis.

Urinary Output. (Table II). Output of free myanesin was followed in 3 dogs by means of periodic urine samples withdrawn through an indwelling Foley bag catheter. To insure urine blanks that give low readings one must obtain dilute urine for the determinations. In this experimental work on dogs it was found that water diuresis, the water being administered by stomach tube the evening before and every 2 or 3 hours throughout the day of the experiment in quantities of 50 to 75 ml per kg of body weight, greatly enhanced the accuracy of the determinations, since an output of light straw-colored urine could thus be insured.

The rate of excretion of free myanesin was found to parallel the concentration of myanesin in the plasma. However, only from 0.133 to 1.93% of the administered dose was detected as free myanesin in the urine; and the excretion of free myanesin was complete when the plasma level reached zero. (Fig. 1). The concentrations of free myanesin obtained in urine are so low, that the failure of urine collected from rabbits, after large doses of the drug, to have any paralytic effect on mice[1] is readily understood.

Output of conjugated myanesin was followed in 4 dogs; in 2 samples were collected by means of the Foley bag catheter, and in two by recovering the urine from the cage as it was normally passed. From Fig. 1 and Table II it can be seen that the excretion of conjugated myanesin persists for 24 hours or more in some dogs, although the greatest quantity is excreted within 3 or 4 hours. From 32 to 42% of the administered dose is excreted in conjugated form in 24 hours. However, the water diuresis necessary for this procedure may have given results somewhat at variance from normal animals.

Conjugation Product. Myanesin bears a chemical resemblance to propylene glycol

which has been shown by Neubauer[4] and confirmed by Fellows *et al.*,[5] to stimulate an increase in the excretion of glucuronic acid (conjugation?). However, glycerol itself and ethylene glycol did not produce such an increase when administered gastrically or hypodermically in rabbits.[5]

Preliminary observations would seem to indicate that myanesin is conjugated with glucuronic acid, at least in part. Neuberg and Schewket[6] have devised a method for the extraction and identification of conjugated glucuronic acid which they claim to be specific. By employing this method of extraction unequivocal reactions were obtained with naphthoresorcinol,[6,7] although inconsistent reactions were obtained with orcinol,[6,7] on urine excreted after the administration of myanesin, whereas similarly treated control urines gave only the weakly positive reactions expected of normal urine.

Summary. A colorimetric method has been presented for the determination of myanesin in plasma and in urine. The rapid decay curve of myanesin in dog plasma explains the brevity of its pharmacological action. In the dog, from 0.1 to 2.0% of the administered dose is excreted as free myanesin; from 32 to 42% of the administered dose is excreted as conjugated myanesin in 24 hours, possibly as the glucuronide.

4 Neubauer, O., *Arch. exp. Path. u. Pharmakol.*, 1901, **46**, 133.

5 Fellows, J. K., Luduena, F. P., and Hanslik, P. J., *J. Pharmacol. and Exp. Therap.*, 1947, **89**, 210.

6 Neuberg, C., and Schewket, O., *Biochem. Z.*, 1912, **44**, 502.

7 Hawk, P. B., and Bergheim, O., *Practical Physiological Chemistry*, Philadelphia, P. Blakiston's Son & Co., Inc., 11th edition, 1937, pp. 68, 658.

16057

The Null Effect of Hemorrhage on Intestinal Absorption of Chloride in the Presence of Sulfate.*

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Goldschmidt and Dayton[1] showed that sodium sulfate increases the absorption of sodium chloride in the large intestine of dogs. Burns and Visscher[2] later demonstrated that the presence of sodium sulfate and certain other anions of the lyotropic series caused the chloride ion, at a concentration below that in the blood plasma, to leave the small intestine and enter the blood against its diffusion gradient.

Van Liere, Northup, and Sleeth[3] noted that isotonic sodium chloride solution was absorbed significantly faster from the intestine of dogs which had sustained a severe hemorrhage (3.2% of their body weight). Since hemorrhage thus simulated the effect of the sulfate ion, it was of interest to study their combined effect.

Methods. Dogs, selected in pairs as nearly alike in weight and age as possible, were fasted 24 hours previous to the experiment. One served as a control and the other was subjected to hemorrhage. Fifteen experiments were performed on a total of 29 animals (one control died).

* Aided by a graant of the Ella Sachs Plotz Foundation.

1 Goldschmidt, S., and Dayton, A. B., *Am. J. Physiol.*, 1919, **48**, 459.

2 Burns, H. S., and Visscher, M. B., *Am. J. Physiol.*, 1934, **110**, 490.

3 Van Liere, E. J., Northup, D. W., and Sleeth, C. K., *Am. J. Physiol.*, 1938, **124**, 102.

TABLE I.
Effect of Hemorrhage on Intestinal Absorption of Chloride in the Presence of Sulfate.

	Control* %	Experimental† %	Difference %	"p"‡
Absorption of chloride	69	81	12	.16
" " sulfate	24	30	6	.15
" " fluid	34	44	10	.11
Final conc. of chloride	0.187	0.138	0.049	.15
" " " sulfate	1.17	1.22	0.05	.20

* 14 animals.
† 15 "
‡ Significant when "p" (according to Fisher) is 0.05 or less.

The experimental animals were etherized and blood equal to 3% of the body weight was withdrawn from the cannulated femoral artery. The artery was ligated and the incision closed. Four hours were allowed for recovery from the ether and adjustment of the circulatory mechanism. During this interim, water was allowed *ad libitum*. The control animals were subjected to the same procedure without, of course, being bled.

Under sodium barbital anesthesia (300 mg/kg intravenously) the small intestine was exposed and practically the entire ileum used for a loop. The same length of loop was used in the control and the bled animal. It was washed out with isotonic glucose solution and filled with a fluid composed of equal parts of isotonic sodium chloride and isotonic sodium sulfate solutions. The resulting mixture contained Na 0.647%; Cl 0.055%, and SO_4 1.278%.

Forty minutes later the fluid remaining in the intestine was removed and the amount carefully measured. An aliquot portion was digested with concentrated HCl and the amount of sulfate determined gravimetrically by precipitation with barium chloride. A second aliquot was used to determine the chloride as sodium chloride by the Van Slyke modification of the Volhard method.

Results and Discussion. The absorption of the chloride ion in the presence of the sulfate was not significantly increased by hemorrhage, although there was a trend in that direction. Since a hemi-isotonic sodium chloride solution was used, it was necessary for absorption to take place against a distinct gradient. Perhaps this explains why hemorrhage did not significantly accelerate the absorption of chloride as it had when isotonic solutions of sodium chloride were used.[3] As previously noted, Goldschmidt and Dayton[1] observed that chlorides were absorbed more rapidly from the colon when a sulfate was present. It has not been shown, to our knowledge, however, that this effect obtains in the small intestine.

Less absorption of chloride and fluid was noted in the control animals. As in our previous absorption studies a fair degree of parallelism was observed between the absorption of the chloride ion and fluid.

At the end of the 40-minute period both the control and experimental animals showed definite chloride impoverishment. This was more pronounced, on the average, in the latter group but, presumably because of the wide individual variation in the several animals, the difference was not statistically significant. In both groups the sulfates showed a definite increase in concentration, because more water than sulfate was absorbed. This increase was not unexpected in the control dogs. In our laboratory it has recently[4] also been shown that a pronounced hemorrhage does not significantly affect the absorption of magnesium sulfate from the small intestine.

Summary. The absorption of chloride, sulfate and fluid from a mixture of equal parts of isotonic sodium chloride and isotonic sodium sulfate solution was studied in dogs which had suffered a hemorrhage of 3% of their body weight.

The percentages of absorption in control

4 Van Liere, E. J., Northup, D. W., Stickney, J. C., and Richard, R. E., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 62.

and experimental dogs were, respectively, 69 and 81 of the chloride; 24 and 30 of the sulfate; and 34 and 44 of the fluid.

In both control and experimental animals the sulfates showed a definite increase in concentration at the end of the experimental period, the chlorides a definite impoverishment. However, the differences between the control and the experimntal animals were not statistically significant.

16058

Influence of Iodinated Trypsin on Blood Coagulation.*

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It has been known for some time that the addition of trypsin accelerates the coagulation of normal blood[1-4] and the blood of hemophilic individuals.[5,6] Intravenously administered trypsin is, however, quite toxic as pointed out by Eagle and Harris.[4] The similarity between the symptomatology resulting from the injection of the enzyme and anaphylactic reactions was observed by Rocha e Silva[7] and these observations have been extended by Dragstedt and his associates.[8-12] Shock resulting from the injection of a relatively small amount of trypsin is indicated by a marked fall in blood pressure.

Tagnon[13] slowly injected trypsin intravenously in hemophilic patients and observed a significant shortening of the coagulation time; however, he has advised extreme caution since the dose most effective on clotting time is very near to that which is quite toxic.

In an earlier report[14] the author pointed out that the iodination of trypsin serves to decrease greatly its hypotensive effect while its proteolytic activity is decreased only about one tenth as much. Relatively large amounts of the iodinated enzyme do not cause the profound fall in blood pressure observed with the same amount of untreated trypsin even with rapid injection.

The present report deals with the influence of iodinated trypsin on the rate of blood coagulation *in vitro*.

Methods. Freshly drawn dog blood was mixed with 3.3 mg of sodium citrate per ml of blood. The plasma was separated after centrifuging for 2 hours at 3200 r.p.m. With short periods of centrifugation the clotting

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11 Rocha e Silva, M., and Dragstedt, C. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 152.

12 Wells, J. A., Morris, H. C., and Dragstedt, C. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 209.

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TABLE I.

Acceleration of Coagulation by Iodinated and Untreated Trypsin. Each tube contained 0.5 ml of plasma and 0.1 ml of 1% $CaCl_2 \cdot 2H_2O$ plus other additions as indicated.

No.	NaCl 0.9% ml	Non-iodinated trypsin 0.1% ml	Iodinated trypsin: 1.6 × 10⁻³ m Eq iodine per mg trypsin	Iodinated trypsin: 1.8 × 10⁻³ m Eq iodine per mg trypsin	Iodinated trypsin: 2.0 × 10⁻³ m Eq iodine per mg trypsin	Avg coagulation time, sec	% of normal coagulation time
1	0.1	0	0	0	0	755	100.0
	0	0.1	0	0	0	68	9.0
	0	0	0.1 ml of 0.3%	0	0	75	9.9
2	0.1	0	0	0	0	221	100.0
	0	0.1	0	0	0	37	16.7
	0	0	0.1 ml of 0.1%	0	0	47	21.2
	0	0	0.1 ml of 0.3%	0	0	35	15.8
3	0.1	0	0	0	0	338	100.0
	0	0.1	0	0	0	47	13.9
	0	0	0	0.1 ml of 0.3%	0	50	14.8
4	0.1	0	0	0	0	475	100.0
	0	0.1	0	0	0	54	11.4
	0	0	0	0.1 ml of 0.3%	0	65	13.7
5	0.1	0	0	0	0	358	100.0
	0	0.1	0	0	0	54	15.1
	0	0	0	0	0.1 ml of 0.1%	95	26.5
6	0.1	0	0	0	0	430	100.0
	0	0.1	0	0	0	65	15.1
	0	0	0	0	0.1 ml of 0.1%	116	27.0
	0	0	0	0	0.1 ml of 0.3%	76	17.7
7	0.1	0	0	0	0	130	100.0
	0	0.1	0	0	0	41	31.5
	0	0	0	0	0.1 ml of 0.3%	44	34.0
	0	0	0	0	0.1 ml of 0.5%	40	30.9
8	0.1	0	0	0	0	127	100.0
	0	0.1	0	0	0	41	32.2
	0	0	0	0	0.1 ml of 0.1%	48	37.8
	0	0	0	0	0.1 ml of 0.3%	44	34.6
	0	0	0	0	0.1 ml of 0.5%	42	33.0

time was considerably less and the accelerating influence of untreated or iodinated trypsin was less apparent. Plasma and the diluent or the enzyme were placed in tubes having inside measurements of 5 x 55 mm and allowed to come to 38°C in a water bath. Calcium chloride was then added, a stopper quickly inserted and the tubes were slowly and uniformly rocked back and forth under the surface of the 38° bath until the air bubble in the tube became stationary and a clot was observed. With these small tubes it was found desirable to begin timing the coagulation when the tubes were first inverted with the calcium chloride present.

The crude trypsin was iodinated under conditions described earlier.[14]

Results. The data of Table I indicate that iodinated trypsin, as well as the untreated enzyme, considerably hastens the coagulation of recalcified plasma. The difference in the degree of acceleration by the untreated and by the iodinated enzyme is similar to the difference in their proteolytic activities described earlier.[14] Approximately the same degree of acceleration by iodinated trypsin can be accomplished by employing 3 to 5 times as much material. However, like the rate of proteolysis, acceleration of coagulation is not directly proportional to the concentration of the enzyme and appreciable acceleration results when iodinated trypsin is used

in amounts equal to the optimum amount of the untreated material.

Further study of the numerous factors to be considered in the use of iodinated trypsin *in vivo* is in progress.

Summary. The coagulation of recalcified plasma, *in vitro,* is hastened considerably by trypsin which has been iodinated sufficiently to greatly decrease its hypotensive effect.

16059

Depth of Penetration of Nebulized Substances in the Respiratory Tree.*

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(Introduced by E. Ogden.)

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To effect a local concentration of penicillin in the pulmonary system, some physicians have been administering nebulized penicillin in the inspired air.

Administering penicillin mist by the usual method of inserting a spray into the oral pharynx uses two routes of administration simultaneously. The mist deposited on the pharyngeal mucosa is absorbed, diluted by the blood stream and distributed throughout the body; also some of the mist may follow the column of inspired air down the respiratory passages to effect a local surface action. Intramuscular injection of penicillin produces higher blood concentrations than is possible by mucosal absorption of the relatively small dosage used in nebulizers. Thus, the only advantage of nebulization is a possible local action on the surfaces of the respiratory passages.

The purpose of this study was to determine the magnitude of concentration and the depth of penetration down the respiratory passages, of a nebulized substance administered with inspired air.

Liquids, such as Lipiodol, pass rapidly to the terminal bronchioles and alveoli. It is known from unpublished war research that aerosol clouds in which the mass median diameter is carefully controlled will penetrate in small concentrations to the alveoli. Dusts, such as silica, over long periods of exposure may penetrate deeply. This study is not concerned with such phenomena, but only with conditions similar to those of aerosol therapeusis, *i.e.,* mixture, with commercial nebulizers, of mist with inspired air in the oral pharynx, and durations of exposure of 15-60 minutes of eupneic breathing.

Nebulized particles in inspired air tend to be deposited against the walls of the respiratory system by settlement due to gravity and by changes in the direction of air flow. The particles tend to be retained in the system by impingement on moist and adhesive surfaces. The deposition due to changes in direction of air flow increases with the velocity of air flow and turbulence, whereas settlement, due to gravity, is only effective where the velocity is low.

Hatch[1] states that maximum air velocities occur in the nasal passages. The mean velocity of the air column in the bronchioles is reduced to 1/1000, and at the entrance of the alveoli is further reduced to 1/100,000 of nasal velocity. Thus the greatest deposition of nebulized particles should be expected in the upper respiratory tree. The air velocity

* Read in abstract, to the Federation of American Societies for Experimental Biology, Chicago, May, 1947.

[1] Hatch, T. F., Behavior of Microscopic Particles in the Air and in the Respiratory System, p. 102, in *Aerobiology,* Moulton, F. R., editor, publication of the A.A.A.S., No. 17, Washington, D.C., 1942.

reaches zero throughout the system for a portion of each respiratory cycle during which period settlement by gravity is possible. However, it is generally accepted in standard texts[2] that respiratory movements do little other than ventilate the respiratory passages down to the finer subdivisions of the bronchioles (Henderson *et al.*[3]). A gas finds its way to the depth of the alveoli only by molecular diffusion. Since nebulized substances are much larger than molecules of a gas, the laws of diffusion do not apply. Nebulized particles, therefore, are transported only when there is an active movement of a column of air, at which time the forces of inertia and impingement causing deposition of such particles are greatest.

An additional factor preventing penetration and retention is the return of some of the particles in the inspired air to the outside atmosphere with expiration. Brown[4] has shown that retention of dust particles is inversely proportional to the respiratory rate for rates below 20 per minute, thus, the slower the respiration the greater the retention. He also concluded that the percentage retention was not affected by volume per respiration, vital capacity or relative humidity of inspired air.

It is often stated that a 5 micron particle will float airborne to terminal air passages. This statement is based on the fact that the terminal bronchioles are 300-400 microns in diameter, and thus it is assumed that a 5 micron particle should have no difficulty in making the passage. This assumption is open to criticism.

Van Wijk and Patterson[5] studied the proportion of particles of different sizes removed from dust-laden air by breathing. They found that 25% of 0.2 micron particles are removed, 80% of 2 micron particles are removed, and 95% of 5 micron particles are removed. They further state that the larger particles are deposited in the larger air passages, thus, the smaller the particle, the less the retention. Bryson and co-workers[6] made photomicrographs of penicillin aerosol reporting the average particle diameter to be 0.54 microns with a range of 0.24 to 1.18 microns.

It would appear from these purely theoretical considerations that large concentrations of aerosol mists will not penetrate deeply into the respiratory tree because

1. The forces of inertia and impingement causing deposition are greatest in the larger branches;
2. The moving air column does not penetrate to the alveoli;
3. The mist will not diffuse as will a gas;
4. There is a return to the outside of some of the mist; and
5. The larger particles which are most likely to be retained are most likely to be deposited in the larger bronchioles.

The literature reveals only one experimental study which separates clearly the factor of pharyngeal absorption from actual penetration of mist. Krueger and co-workers[7] confined an adult monkey in an atmosphere of dilute India ink for 60 minutes. On gross examination, following autopsy, carbon was found in large amounts in the nasal passages, larynx and stomach, in smaller amounts in the trachea and bronchi. Microscopic sections showed that some inspired material had penetrated into the alveolar spaces. The following experiments were undertaken to investigate the apparent lack of agreement between the theoretical considerations and the experimental result.

Methods. The depth of penetration and magnitude of concentration of various nebulized substances in the respiratory tree was determined experimentally in 26 animals (2

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7 Personnel of Naval Laboratory Research Unit No. 1, Command of Krueger, A. P., and Lyons, W. R., *Am. J. Med. Sci.,* 1944, **207**, 40.

TABLE I.

1	2	3	4	5	6
Angle of bend in glass tube	No bend	one 30° angle	one 90° angle	one 120° angle	two 20° angles
% of mist removed from air column	defined as 0%	30-35%	75-80%	90-95%	50%

mice, 11 rats, 2 rabbits and 11 dogs). A commercial nebulizer ("glaseptic"—Parke-Davis & Co.) connected to a half-liter pressure bottle was used to obtain a fine mist of diluted India ink (1:1 or 1:5 aqueous dilution), gentian violet (1%-5% aqueous solution) or ferric ammonium sulfate (1%-5% aqueous solution). It was found that a hand bulb gave a more sustained mist with smaller droplets than did oxygen at 8 to 10 liters per minute; so the former was used exclusively after the first 4 animals studied.

The size of the nebulized droplets sprayed from the pressure bottle was directly observed by comparison with red cell smears to include particles as small as 0.5 microns with 80% being 1 to 3 microns in diameter.

The animals were lightly anesthetized with pentobarbital sodium (Nembutal). The mists were introduced to the respiratory tree by a glass tube placed in the oral-pharynx, or in some animals by a tracheal cannula placed in the same axis as the trachea. The rate and depth of respirations were noted. The animals were exposed to the mists for periods of from 15 to 60 minutes. They were immediately sacrificed and gross and microscopic observations were made.

Preliminary experiments on the action of suspensions passing through a system of branching glass tubes demonstrated the removal of particulate matter from the air column at the points where the direction of the air column changed. The amount of nebulized suspension of dilute India ink passing through a straight tube was accepted as a measure of 0% removal (Table I). As the angle of bend in the tube increased, and as the number of bends in the tube increased, there was an increase in the removal of particulate matter from the air column. The carbon was deposited largely on the walls opposing flow at the bends in the tubing.

Results. 1. The first group of one rat and 2 mice was not anesthetized nor was an oral cannula used. The animals were placed in an atmosphere of a mist of 50% India ink for 60 minutes. Carbon particles were found in the nasopharynx and stomach of each animal. No carbon was seen grossly or microscopically in the larynx, trachea, bronchi, bronchioles, or alveoli.

2. Because the nasal passages appeared to be removing most of carbon from the inspired air in the first group, a second group of 7 rats and 2 rabbits, lightly anesthetized, was exposed to India ink, gentian violet or ferric ammonium sulfate solutions for periods of from 15 to 30 minutes. The mist was introduced to the inspired air by an oral cannula. The nose was occluded and the tongue pulled forward to insure mouth breathing.

Particles were seen grossly to be present in rather large quantities in the oral pharynx and larynx of all animals. Below this point the nebulized substances were noted in small quantities, mostly at the point of branching of the bronchi. Microscopic sections indicated no evidence of foreign material in the alveoli. Only one cell out of every one or two thousand lining the bronchi showed any evidence of foreign material.

3. Because of the negative results in rats and rabbits, a third group of 4 dogs was exposed to India ink suspensions (1:5 aqueous dilution) and ferric ammonium sulfate (1% aqueous solutions) with exposures of from 30 to 60 minutes. The results were essentially the same as with the second group of animals except that in two animals there were minute particles in the larger bronchioles which may have been either artifact or inspired material.

4. In order to by-pass the screening of the inspired air by the pharynx and larynx, the mist was directly introduced through a tracheal cannula into a rat and 2 dogs. This fourth group of animals showed tracheobronchial distribution similar to the other groups, but

larger quantities of carbon and iron were deposited. No particulate matter was demonstrated in the smaller bronchioles or alveoli.

5. Because a satisfactory microscopic picture—for a comparison control—of the deposition of carbon, dye or iron in the alveoli had not yet been produced, an attempt was made to force particulate matter into the alveoli. The lungs and trachea were removed in one block from 2 dogs and 2 rats and were artificially inflated by the nebulizer. A free flow of mist was insured by cutting the tip from one of the lower lobes. The lungs were inflated and deflated beyond physiological limits for a period of 15 minutes. Microscopic sections indicated only a minute amount of foreign material in the bronchi. None of the alveoli was seen to contain foreign material. Even those alveoli adjacent to the free flow of mist at the cut tip were free of particulate matter.

6. The breathing of one dog was stimulated by an intermittent mixture of CO_2 with the inspired air. Dilute India ink mist was introduced into the oral pharynx for a period of 30 minutes. The gross and microscopic distribution was essentially the same as in dogs during the eupneic breathing. No carbon was distributed in the smaller bronchioles or alveoli.

7. Abramson[8] has suggested that 25% glycerol be added to the carbon suspension to lower vapor pressure and thus make a more stable mist. Two dogs were exposed to a glycerol mist containing carbon for 30-minute periods. There were tracheobronchial depositions of carbon in larger quantities than when aqueous suspensions were used. In a dependent lobe of one dog there was a sharply localized area 2 cm in diameter in which carbon was deposited in the alveoli. However, in this dog there was an excess of free flowing fluid in the oral pharynx and the dog coughed and "gurgled" several times during the procedure. It is believed that this localized alveolar deposition was due to aspiration and flow rather than to inspiration of nebulized mist.

Discussion. A careful search of the literature fails to bring to light any actual experimental confirmation of Krueger's experiment[7] in which the factor of pharyngeal absorption is clearly separated from the factor of inspiratory penetration.

Using nebulized sulfonamide solutions, Mutch[9] found that only 7 to 14 percent of the drug leaving the nebulizer reached the blood stream. Later, working with nebulized penicillin, Mutch and Rowell[10] found that only 25% of the mist was absorbed into the blood stream. Neither of these studies determined what proportion of the blood concentration was due to pharyngeal absorption. We have been able to demonstrate only a minimal penetration to the larger bronchi, and a questionable penetration of nebulized substance into the deeper respiratory passages.

Although penicillin aerosol may be adequate for effecting some degree of pharyngeal absorption, it does not yield adequate surface action on the respiratory passages. It is the impression of the authors that the concentration of the substance on the surfaces of the trachea, bronchi and bronchioles would not be sufficient for therapeutic effectiveness.

Summary. 1. An attempt has been made to determine the depth of penetration, and the amount of deposition in the respiratory system of nebulized dilute India ink, aqueous gentian violet solution and aqueous ferric ammonium sulfate solution in 2 mice, 11 rats, 2 rabbits and 11 dogs.

2. The substances were found to be deposited in decreasing amounts in the oropharynx, larynx, bifurcation of the trachea, the bronchi and bronchioles of the animals. The amounts of deposited substances in the bronchi and bronchioles were so small as to make microscopic detection difficult. In no case was any nebulized substance found to have reached the alveoli.

3. There is no evidence to support the theory that pulmonary lesions can be treated by topical application with commercial nebulizers.

8 Abramson, H. A., *Ann. Allergy*, 1946, **4**, 440.

9 Mutch, N., *Lancet*, 1944, **2**, 775.

10 Mutch, N., and Rowell, R. E., *Lancet*, 1945, **1**, 650.

16060

Effect of Urethane on Transplanted Leukemia of Ak Mice.*

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Haddow and Sexton,[1] investigating the effect of urethanes on human malignant tumors, reported that they observed a fall in the leucocyte counts. Prompted by this Paterson, Haddow, Thomas, and Watkinson[2] treated 32 cases of human leukemia, 19 myeloid and 13 lymphatic, with urethane, (ethylcarbamate). In both types of leukemia they observed a decrease in the total white blood cell count with a return towards normal of the differential pattern. This was accompanied by decrease in the size of the spleen and lymph nodes and a rise in the hemoglobin level. They considered the effects to be comparable to those obtained with deep x-ray therapy. There was no evidence that permanent benefit resulted from the therapy. Engstrom, Kirschbaum, and Mixer[3] tested the effect of urethane on the transplanted myelogenous leukemia in F strain mice. They found that just sub-lethal doses were effective in reducing the white blood cell counts to normal but did not eradicate the infiltration of the tissues. Smaller doses had a less marked effect on the counts. Kirschbaum and Lu[4] demonstrated later that a single anesthetic dose of urethane decreased the number of mitotic figures in marrow myeloid cells and reduced the total number of circulating white cells, especially myeloblasts, in transplanted myeloid leukemia of F strain mice.

Mice of the Ak strain have a very high incidence of spontaneous leukemia. About 75% develop the disease after the age of 6 months. The intravenous injection of leukemic cells from diseased older animals produces progressive leukemia in 100% of younger animals. The leukemic tissue is cut into small pieces with scissors and immersed in Tyrode's solution. The larger particles are allowed to settle leaving a suspension of cells in the supernatant fluid. The concentration of cells in the suspension is determined by counting in a hemacytometer. Dilution is usually necessary until the required concentration is reached. The injection into the tail vein of about 40,000 cells suspended in 0.10-0.15 cc of solution produces leukemia in all of the young animals in about 2½ weeks and death occurs in 3 to 5 weeks.

Twenty Ak animals† less than 5 months old were selected and divided into 3 groups. Five received the cell suspension only; 10 received cell suspension and were treated with urethane; and 5 were treated with urethane only. It had been determined previously that the maximum dose tolerated over a 5-week period by mice weighing 25-30 g was 7.5 mg injected subcutaneously twice daily. This dosage was used. White blood counts and differential counts were done once weekly for 2 weeks and then twice weekly. At autopsy sections of the liver, spleen, and bone marrow were taken for histologic study.

The white blood cell counts of the animals which were treated with urethane and were not injected with cell suspension did not show any variation from the normal. The average and individual total white blood cell counts of the other two groups are shown in Fig. 1.

* This research was made possible by a grant from the Price McKinney Memorial Fund.

1 Haddow, A., and Sexton, W. A., *Nature* (London), 1946, **157**, 500.

2 Paterson, E., Thomas, I. A., Haddow, A., and Watkinson, J. M., *Lancet*, 1946, **250**, 677.

3 Engstrom, R. M., Kirschbaum, A., and Mixer, H. W., *Science*, 1947, **105**, 255.

4 Kirschbaum, A., and Lu, C. S., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 62.

† The Ak mice used in this experiment were from a colony developed from breeders given to us by Dr. Jacob Furth.

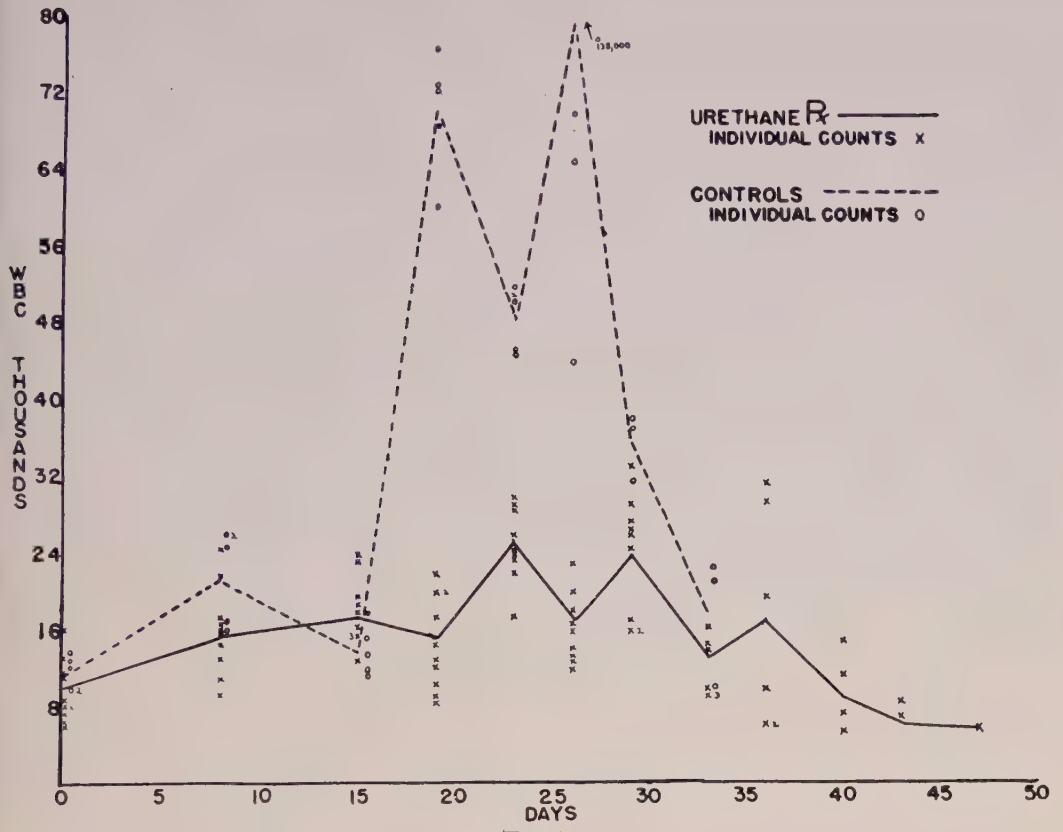

FIG. 1.

Average and individual white blood cell counts of the urethane-treated group and the control group are shown. Only a very slight rise above normal occurred in the urethane group.

In Fig. 2 the averages of the differential counts are plotted. The change in counts of the animals which were given cell suspension and not treated with urethane was entirely characteristic of the transplanted disease. Evidence of leukemia was manifest in the peripheral blood on the 19th day, at which time primitive blast cells were first seen. The leukemia was at its peak on the 26th day and all the animals were dead by the 34th day. The drop in white blood cell count shortly before death is usually seen. The treatment with urethane had a striking effect on the course of the transplanted leukemia. The total and differential counts showed no change from the normal until the 23rd day at which time there was a slight rise above normal and a few blast cells appeared. The slight rise was maintained until the 40th day at which time the total counts fell to within normal limits. A small percentage of blasts, however, persisted until the death of the last animal on the 48th day. The urethane had the effect of delaying the appearance and greatly diminishing the number of white blood cells in the peripheral blood.

The histologic changes in the control group not treated with urethane were typical of the transplanted disease. The infiltration of the organs lagged behind the development of leukemia as manifested by changes in the peripheral blood. The 2 animals which survived only 22 and 24 days showed only moderate infiltration. The 3 which survived 31, 31, and 34 days showed maximal infiltration. The animals treated with urethane also developed infiltration in the organs but this occurred later and to a less marked degree than in the untreated animals. One mouse that survived 27 days showed no infiltration.

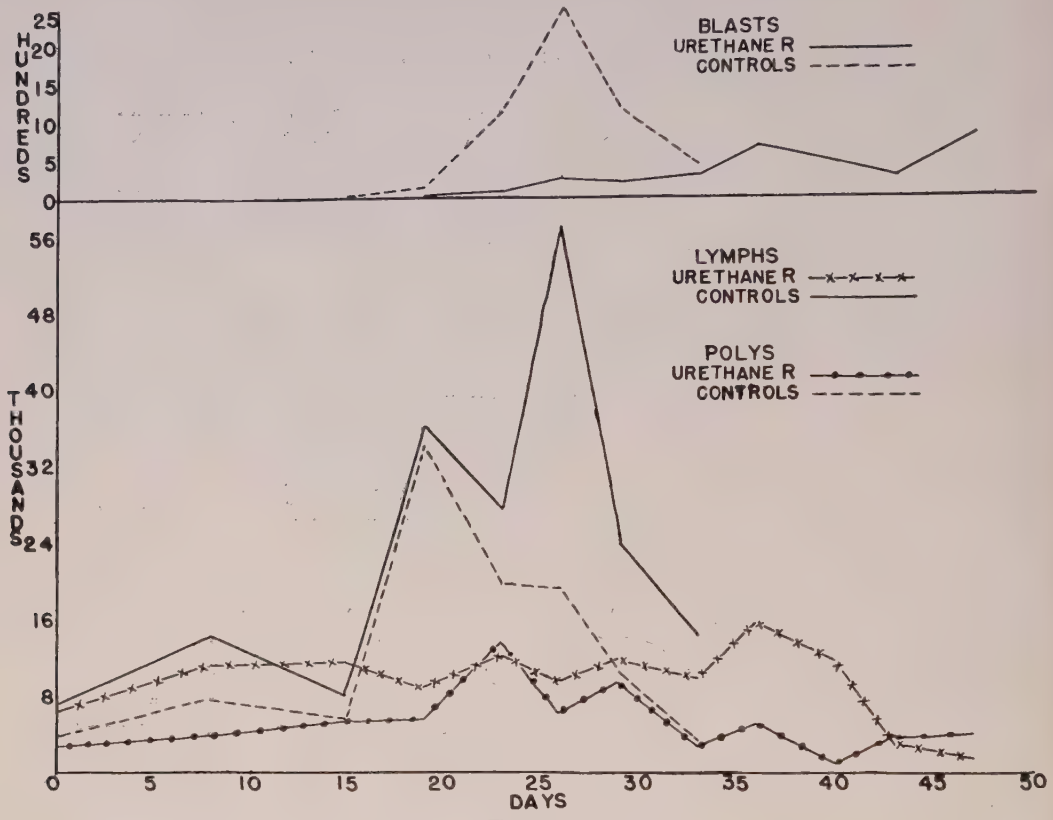

FIG. 2.

Average differential counts of the urethane-treated group and the control group are shown. Primitive blast cells appeared later and in smaller numbers in the urethane group.

Another that survived 29 days showed minimal infiltration in the spleen and one small focus of leukemic cells in the liver. In the animals that survived 32 to 48 days there was moderate infiltration which was much less extensive than that present in the untreated animals surviving 31 to 34 days. There was a general tendency to weight loss which was not extreme in any one group and was not more or less marked in any one group.

The urethane treatment increased the survival time of the animals injected with cell suspension. The average survival time of those not treated with urethane was 28.4 days, of those treated with urethane 36.0 days. This is statistically significant. P equals 0.05.[5] Of the 5 animals which were treated with urethane and not injected with cells one died on the 19th day and one on the 41st. The other 3 were killed on the 50th day. It is probable that the dose of urethane was toxic and was approaching a lethal dose.

Conclusions. The administration of urethane to Ak mice with transplanted leukemia caused a delay in the appearance of immature cells in the peripheral blood and prevented a marked increase in total white blood cell count and numbers of immature blast cells. Infiltration of organs occurred later and to a less marked degree. The average survival time of the animals was increased and this increase was statistically significant. In order to secure these effects a dosage of urethane was used which was just sub-lethal. It is probable that the drug itself was partly responsible for the death of the treated animals.

[5] Fischer, R. A., *Statistical Methods for Research Workers,* Oliver and Boyd, Edinburgh, 1934.

16061

Alloxan Diabetes in Hypophysectomized Rats.*

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Since diabetes can be produced in certain animals by the injection of alloxan[1,2,3] or by the injection of crude anterior pituitary extract,[4–7] this investigation was undertaken to determine whether alloxan produces its diabetogenic effect through the pituitary gland.

For many years an association between the pituitary and diabetes mellitus has been suggested by (1) the high incidence of diabetes and glycosuria among acromegalics,[8] (2) the production of experimental diabetic animals by the injection of crude anterior pituitary extract,[4–7] (3) the marked hypersensitivity to insulin exhibited by hypophysectomized animals,[9] (4) the amelioration of the diabetes in depancreatized dogs or toads following hypophysectomy,[10,11] (5) the suggestion of pituitary overactivity in children[12a] in the few months preceding the onset of diabetes as evidenced by rapid dental and osseous development beyond that expected for their age.

Although both alloxan and crude anterior pituitary extract (A P E) produce islet cell destruction and diabetes when injected into dogs, there are some striking differences in their action, the most outstanding of which are as follows:

(1) The injection of APE produces diabetes only in the dog and in the partially depancreatized rat or cat,[13] whereas alloxan has already been shown to produce diabetes in the dog, rat, cat, rabbit, pigeon, monkey, sheep and turtle.[12b]

(2) Hyperglycemia is imperative for the production of diabetes with APE.[13] The use of insulin, phlorhizin or a low carbohydrate diet which prevents hyperglycemia also prevents the development of diabetes. Alloxan however produces islet cell necrosis and diabetes irrespective of hyperglycemia.[14]

(3) Alloxan produces islet cell destruction and diabetes within 24 hours[15] after injection of a single dose, whereas APE must be injected daily for several days or weeks.[4]

(4) Pancreatic islet lesions produced with a single diabetogenic dose of alloxan show degenerative changes from the start with destruction of all or nearly all of the beta cells within 24 hours.[15] No similar rapid islet destruction follows APE but rather a gradual degranulation with the development of extensive hydropic degeneration of the beta cells.[16]

The production of diabetes by the injection

* We are indebted to the American Cyanamid Company for a research grant.

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3 Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **2**, 384.

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11 Houssay, B. A., and Biasotti, A., *C. R. Soc. de Biol.*, 1930, **105**, 121.

12 Joslin, E. P., Root, H. F., White, P., Marble, A., and Bailey, C. C., *The Treatment of Diabetes Mellitus*, 8th Edition, Philadelphia, Lea and Febiger, 1946, (a) p. 743, (b) p. 178.

13 Lukens, F. D. W., *Yale J. Biol. and Med.*, 1944, **16**, 301.

14 Goldner, M. G., and Gomori, G., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 73.

15 Bailey, O. T., Bailey, C. C., and Hagan, W. H., *Am. J. Med. Sci.*, 1944, **208**, 450.

16 Duff, G. L., *Am. J. Med. Sci.*, 1945, **210**, 381.

of repeated small doses of alloxan produces some degranulation and hydropic degeneration in the beta cells but not to a degree comparable to that following the administration of APE.[15]

In view of these findings, it seemed appropriate to investigate whether the presence of the pituitary is essential for the production of alloxan diabetes. This report deals with hypophysectomized animals injected with alloxan.

Materials and Methods. White rats of the Hisaw strain, obtained from the Biological Laboratory of Harvard University, were used. They were maintained on Purina Fox-Chow and water. After hypophysectomy glucose water was also made available at all times. (See Discussion.)

Hypophysectomy was done by one of us (C.C.F.) using a parapharyngeal approach through the base of the skull, the technique of which has been described in detail elsewhere.[17]

Approximately 2 weeks after operation, a control micro blood sugar determination (Folin-Malmros method) was done on blood obtained by cutting off the tip of the rat's tail. Blood samples were obtained near the middle of the day throughout the experiments and no attempt was made to withhold food. (See Discussion.) A freshly prepared 5% solution of alloxan (Eastman Kodak Company) was then administered subcutaneously in an initial dose of 150 mg per kg in 15 rats and 100 mg per kg in 4 rats. When no hyperglycemia occurred, the initial dose was repeated or the dose was increased by 25 or 50 mg per kg. Animals were considered diabetic when the blood sugar exceeded 200 mg % 24 or more hours after the injection of alloxan.

In evaluating the completeness of the hypophysectomy the chief clinical criterion used was failure to gain in weight. At autopsy a careful search was made for remnants of the pituitary and, if any were found, the animal was omitted from the series.

In most instances the animals were killed with ether, and autopsied immediately, the fresh tissues being fixed usually in Bouin's and Helly's fluid. In most cases sections were made of all the important organs. The pancreas was examined in all the rats included in the final series. Gomori's chrome-alum hematoxylin stain[18] proved to be most satisfactory for differentiating the beta and alpha cells in the islets of Langerhans.

Results. The final series consisted of nineteen rats. These were divided into groups on the basis of their clinical behavior, as follows:

Group A—Diabetes; animals sacrificed soon after onset	5 rats
Group B—Diabetes; animals allowed to survive for months	3 "
Group C—Transitory diabetes	7 "
Group D—Apparently resistant to alloxan in doses given	4 "

Group A—*Diabetes; Animals Sacrificed Soon After Onset.* This group comprises 5 rats sacrificed after being diabetic for periods varying from 2 to 28 days. Four of these animals had blood sugar levels over 400 mg %, after a single dose of 150 mg alloxan per kg. The fifth animal received doses of 100, 125, and 150 mg per kg at approximately weekly intervals without obvious effect but developed hyperglycemia after 175 mg per kg was given. The incidence of diabetes in this group does not appear to differ from that in normal rats injected with alloxan. (See Discussion.)

Group B—*Diabetes; Animals Allowed to Survive for Months.* The 3 rats in this group remained diabetic for relatively long periods, one for 2½ months, and 2 for 8½ months. One of these required a dose of 300 mg per kg before diabetes was definitely established. All 3 animals showed some fluctuation in blood sugar levels, all having occasional blood sugar values below 200 mg %. This point is commented upon in the discussion below. One animal developed cataracts in both eyes.

Group C—*Transitory Diabetes.* Six of the 7 animals in this group showed a single high blood sugar a few days following the first injection of alloxan and then one or more

[17] Franseen, C. C., Brues, A. M., and Richards, R. L., *Endocrinology*, 1938, **23**, 292.

[18] Gomori, G., *Am. J. Path.*, 1941, **17**, 395.

blood sugars below 200 mg %. Three of the rats later responded to a larger dose of alloxan with a blood sugar over 200 mg %, but all 3 again had a subsequent blood sugar less than 150 mg %. In other words, the blood sugar levels showed wide fluctuations and a striking tendency to revert to normal.

Group D—*Animals Apparently Resistant to Alloxan.* The 4 animals in this group were given from 2 to 5 injections of alloxan, 2 of them receiving a maximum dose of 200 mg per kg and the other 2 a maximum dose of 300 mg per kg without developing hyperglycemia. It is noteworthy that one animal showed apparently complete loss of beta cells from the islets of Langerhans and another showed an acute degenerative change in the beta cells probably due to the last injection of alloxan.

Pathological Findings. In 2 animals (one from Group B, one from Group D), the histological preparations were unsatisfactory owing to postmortem change. The islets of Langerhans in the remaining 17 rats showed loss of beta cells in varying degree, with apparent complete loss of beta cells in the majority, the islets being composed of alpha cells staining pink with the Gomori chrome-alum hematoxylin method. A few surviving beta cells were present in some islets of 2 rats in Group A, one in Group C, and one in Group D. No definite necrosis or other lesion of organs such as liver, kidney, and adrenal was noted. The expected findings in the hypophysectomized animals—*e.g.,* atrophy of gonads, adrenals, and thyroid—were also present.

Discussion. Hypophysectomized rats fail to grow and their fur remains fine and soft. Their weight tends to remain constant and they show an abnormal response to both insulin and to adrenalin. These animals show marked hypersensitivity to insulin and respond to about one-tenth the dose of insulin required to act in normal animals.[19] Adrenalin produces less hyperglycemia than it does in the intact animal. Since hypophysectomized rats show a marked tendency to hypoglycemia and since the administration of alloxan is followed by a transitory hypoglycemia, especial care must be taken to prevent fatal hypoglycemia in hypophysectomized rats given alloxan. For this reason, glucose water was made available.

Perhaps the most striking feature of the experimental results is the large number of animals showing diabetes which was either transitory or fluctuating. These included not only the rats in Group C (transitory group), but also the 3 rats in Group B (long duration), all of which showed one or more normal blood sugars during their course. In other words, even in the permanently diabetic hypophysectomized animal, the blood sugar showed a definite tendency to revert to normal.

In unoperated animals made diabetic with alloxan, a certain number may be expected to exhibit a transitory course, but the fluctuations were not so striking as in the present series. In a previous series of 50 control rats given a dose of 150 mg in this laboratory, the results were classified as follows:

	%
Permanent diabetes	58
Transitory diabetes	12
Resistant to 150 mg	22
Died before first blood sugar	8

In this control group the rats exhibiting permanent diabetes showed sustained high blood sugar levels, without reversion to normal, while those classed as transitory showed high blood sugar levels for a week or so, the subsequent ones being within the normal range. In other words, the striking fluctuations seen in the hypophysectomized animals were not present in the controls.

It is also noteworthy, as remarked above, that all the animals in which good histological preparations were obtained showed destruction, to a greater or less degree, of the beta cells in the islets of Langerhans; *i.e.* the anatomical lesion was present regardless of the apparently mild course of the diabetes.

The phenomenon of amelioration of diabetes following hypophysectomy is, of course, not new, being well known in the "Houssay animal", where hypophysectomy is followed

[19] Russell, J. A., *Physiol. Rev.,* 1938, **18**, 2.

by improvement in the diabetes produced by pancreatectomy. The results of the present experiments are, therefore, not surprising and it would seem that there is no more evidence that alloxan acts through the pituitary than there is that pancreatectomy exerts its effect in this way.

The general question of the relation of the pituitary to carbohydrate metabolism is well discussed by Russell,[19] Houssay,[20] and Lukens.[21]

A preliminary report on the production of alloxan diabetes in hypophysectomized rats was published from this laboratory in 1945.[22] As far as we are aware, the only other reports on alloxan diabetes in hypophysectomized animals are those of Kirschbaum *et al.*,[23] of Duff and Starr,[24] and of Gaarenstroom.[25]

Kirschbaum, Wells and Molander[23] found that hypophysectomized rats injected with alloxan developed severe hypoglycemic convulsions but no experiments were carried beyond 6 hours and no histological sections were reported.

Duff[16] describes unpublished observations by Duff and Starr in which hypophysectomized rats were given alloxan and some survived the initial hypoglycemic stage. Duff states that "although histologic studies showed destruction of the islets of Langerhans quite as extensive as in intact animals, there was no evidence of diabetes during the periods of survival. The blood sugar remained at more or less normal levels, but there were irregular fluctuations somewhat above and below the normal limits."

Gaarenstroom[25–27] reported that extirpation of the hypophysis in animals already made diabetic with alloxan led to a marked decrease or even total disappearance of glycosuria and a fall in blood sugar, if the animals were fasted. After administration of sugar the blood sugar rose again to high levels.

Conclusions. 1. Diabetes was produced by the injection of alloxan in 15 of 19 hypophysectomized rats. 2. The diabetes so produced tended to be either transitory or fluctuating in severity and it is suggested that this phenomenon represents the "Houssay effect" seen in depancreatized hypophysectomized dogs. 3. Extensive loss of beta cells in the islets of Langerhans was noted in all animals in which satisfactory histological preparations were obtained. The presence of hypophysis is therefore not necessary for the production of what appears to be the essential lesion in alloxan diabetes.

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23 Kirschbaum, A., Wells, L. J., and Molander, D., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 294.

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16062

Effect of Anoxic Anoxia on Bile Secretion in the Rat.

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Studies on the effect of anoxic anoxia on the absorption of fat from the alimentary tract of rats have shown that the amount of fat absorbed by animals subjected to partial pressure of oxygen of 63 mm and 53 mm Hg (8.35 and 7.03% oxygen, respectively) was significantly less than for control animals.[1] This cannot be explained on the basis of a prolonged gastric emptying time since it was found that rats fed corn oil showed an initial acceleration of the emptying of the stomach on exposure to diminished oxygen tension.[2]

Glikson and Rubel[3,4] reported that exposure of dogs with a biliary fistula to pressures equivalent to altitudes of 6,000 and 8,000 m (9.78 and 7.68% oxygen, respectively) resulted, as a rule, in a decrease in the volume of bile secreted with an increase in the content of organic solids and the viscosity. To a lesser degree and less constantly there was an increase in the bile acid content. They attributed the changes to oxygen starvation of the hepatic cells. Schnedorf and Orr[5] found that increasing degrees of anoxemia produced by inhalation of 15, 10, and 5% oxygen in nitrogen resulted in a marked and progressive decrease below normal in the flow of bile in nembutalized dogs.

Since it is generally recognized that bile salts play an important role in the digestion and absorption of fat, the decreased rate of fat absorption observed in rats subjected to reduced barometric pressure might reasonably result from a diminished flow of bile. It was considered of interest, therefore, to investigate the effect of anoxic anoxia on the secretion of bile salts in the rat.

Methods. Young adult albino rats were found to be the most suitable for the experiments. Using the bile fistula technique of Harrington, Greaves, and Schmidt,[6] the bile duct was anastomosed to the vas deferens by means of a stainless steel cannula about the size of a No. 24 hypodermic needle. Thus the bile was completely excluded from the intestinal tract and excreted in the urine instead. Six rats were successfully operated by this technique and remained in apparent health for a period of 3 to 5 months. The low-fat diet recommended by Greaves and Schmidt[7] for bile fistula rats was fed, with the exception that meat meal supplemented with 0.25% cod liver oil concentrate was substituted for fish meal which was unavailable at the time. After the rats had recovered from the operative procedure, they were exposed to simulated high altitude for 4-hour periods in individual metabolism cages. At the end of each period the volume of urine was measured and analyzed for its content of bile salts. Exposure to altitude was carried out not more often than once a week to avoid the possibility of acclimatization. Alternating with exposures to altitude control experiments were performed. In the early experiments the rats were subjected to a simulated altitude of 28,000 ft. (53 mm Hg oxygen tension). This resulted in the death of two animals, consequently in the subsequent ex-

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2 MacLachlan, P. L., Proc. Soc. Exp. Biol. and Med., 1946, **63**, 147.

3 Glikson, E. B., and Rubel, V. M., *Arch. sci. biol.* (U.S.S.R.), 1940, **58**, 76.

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6 Harrington, F. G., Greaves, J. D., and Schmidt, C. L. A., Proc. Soc. Exp. Biol. and Med., 1936, **34**, 611.

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TABLE I.
Effect of Anoxic Anoxia on Excretion of Bile Salts and Urine in 6 Bile Fistula Rats. (Average values for four-hour control and experimental periods.)

	No. of Exp.	Bile salts, mg	Urine vol., cc	Corr. coefficient bile salts/urine
Control	32	0.37	2.3	—0.4
Anoxia	32	0.34	3.9	+0.3
Std. Dev.		0.107	1.575	
P (Fisher's)		>0.2	<0.001	

periments the altitude was reduced to 24,000 ft. (63 mm Hg oxygen tension).

The urine was analyzed for its content of bile salts by the procedure of Morrison and Swalm[8] and Morrison.[9] This method was found to give satisfactory results on known solutions of cholic acid, as well as urine samples containing bile supplemented with known amounts of cholic acid.

Results and Discussion. The data, Table I, show that there was no significant difference in the amount of bile salts excreted by bile fistula rats when subjected for 4-hour periods to diminished oxygen tension as compared to control periods. In agreement with the findings of Stickney,[10] there was a statistically significant increase in the output of urine as a result of exposure to simulated altitude. Since there was no correlation between the amount of bile salts and the volume of urine excreted, the values for each were expressed in terms of the amount excreted per 4-hour period.

The range in the volume output of urine was 0.8 to 5.0 cc for the control periods, and 1.2 to 7.7 cc for the periods of exposure to altitude. Apparently the urine, irrespective of its volume, flushed out the bile which had collected in the urethra.

Although the amount of bile salts excreted during the control and experimental periods varied considerably, (0.15 to 0.62 mg and 0.16 to 0.68 mg respectively), the output over a 2- to 3-month interval did not show any noticeable decrease with increasing age of the chronic biliary fistula, as found by Boyd in dogs.[11]

Haney, Roley, and Cole,[12] as a result of studies on dogs with Thiry Vella loops, suggested that bile salts may play an important role in the normal regulation of the propulsive movements of the small intestine. Ackerman, Curl and Crandall,[13] however, working with bile fistula dogs found that in general their data did not lend support to the concept that bile salts are an important factor in the regulation of small intestine motility. Since in rats anoxic anoxia was found to be without effect on the secretion of bile salts, a changed motility of the small intestine as a result of deficient bile secretion cannot be considered of importance as far as the effect of anoxia on fat absorption is concerned.

Summary. No significant difference was found in the amount of bile salts excreted by 6 bile fistula rats when exposed for 4-hour periods to decreased oxygen tension (63 mm and 53 mm Hg) as compared to control periods. There was a statistically significant increase in the volume output of urine as a result of exposure to simulated altitude. No correlation existed between the amount of bile salts and the volume of urine excreted.

[8] Morrison, L. M., and Swalm, W. A., *J. Lab. Clin. Med.*, 1940, **25**, 739.

[9] Morrison, L. M., *J. Lab. Clin. Med.*, 1943, **28**, 1503.

[10] Stickney, J. C., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 210.

[11] Boyd, E. M., Earl, T. J., Jackson, S., Palmer, B., and Stevens, M. E. T., *Am. J. Physiol.*, 1945, **145**, 186.

[12] Haney, H. F., Roley, W. C., and Cole, P. A., *Am. J. Physiol.*, 1939, **126**, 82.

[13] Ackerman, R. F., Curl, H., and Crandall, L. A., Jr., *Am. J. Physiol.*, 1941, **134**, 32.

16063 P

Cross Transfusion as a Means of Determining Toxic Factors in Blood from Burned Animals.

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(With the technical assistance of Mr. Everett Hoppe.)

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Although numerous methods of transfusion of blood from one animal to another have been utilized in an effort to test for toxic products, most of them fail to utilize principles which might prevent dilution of the toxins. When testing for a toxin possibly arising from an extremity removal of blood from a vein in the neck or another extremity would obviously obtain blood in which any toxin would be greatly diluted. In efforts to test for a possible toxin in burned hind extremities of a dog, we resorted to cross transfusion in which 100 cc of blood per kg of body weight was removed

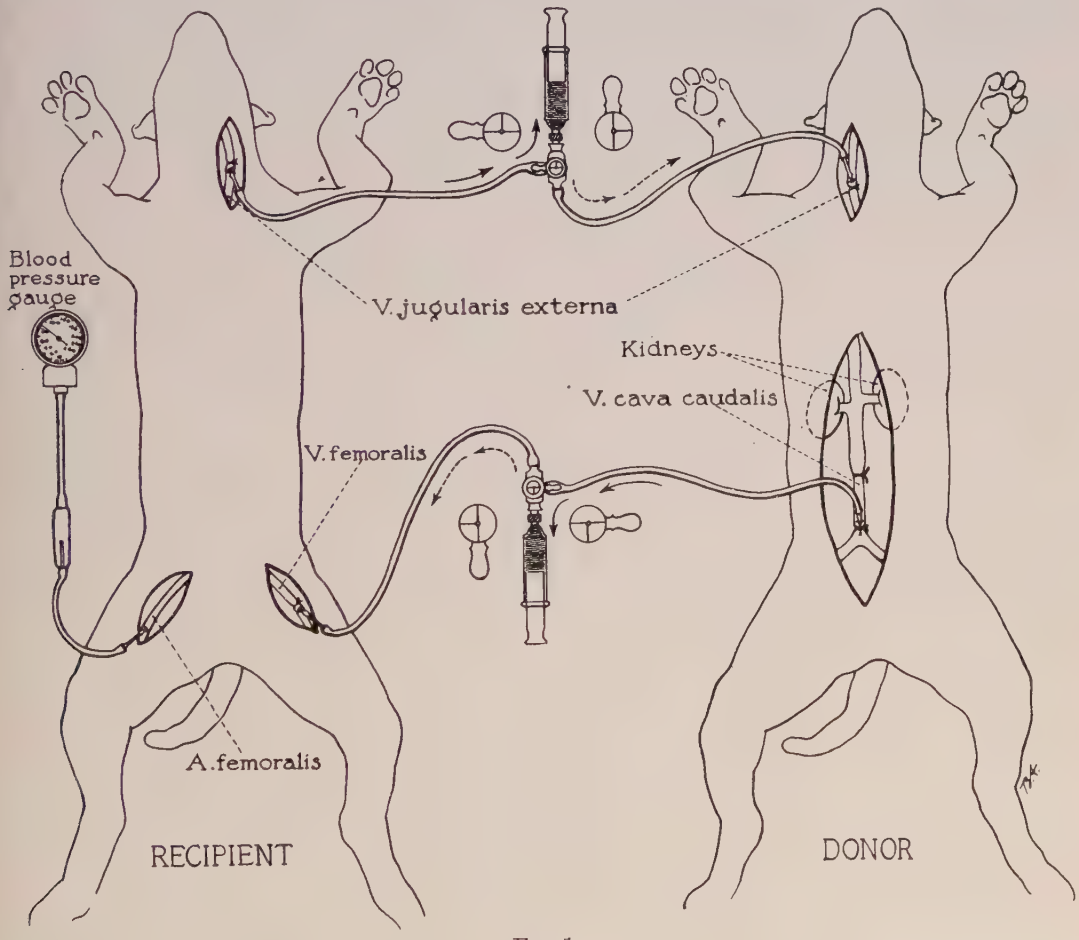

FIG. 1.

In our cross-transfusion experiments blood was removed from the vena cava of the animal (donor) burned on the lower extremities and distal torso; it was injected into the femoral vein of the normal animal (recipient). Blood was removed from the jugular vein of the normal animal and injected into the jugular vein of the burned animal.

TABLE I.
Mortality Rate, Blood Pressure, and Pulse Changes in Animals Receiving Blood from Normal Control Animals as Compared to Animals Receiving Blood from Burned Animals.

	Receiving blood from normal animals	Receiving blood from burned animals
Mortality rate	16.6%	27.7%
Avg drop in blood pressure	19.35 mm Hg	41.83 mm Hg
Avg rise in pulse rate	12.9 beats per min	14.8 beats per min
Total No. of experiments	30	18

over a 25-minute period from the distal vena cava of the burned animal (donor) and transfused into the jugular vein of the normal animal (recipient), as illustrated in Fig. 1. This obtained blood directly from the area of the burn which was produced by immersion of the distal portion of the animal for 30 seconds up to the third thoracic vertebra in vegetable oil heated to 120°C. All animals used in these experiments were anesthetized with 33 mg sodium pentobarbital per kg of body weight injected intraperitoneally; ether was used in a smaller series not reported herein. Blood from the 2 animals being cross-transfused was matched previously to rule out incompatibility. To facilitate transfusion, 5 mg heparin per kg of body weight was injected intravenously into the animals. The cross-transfusions were performed 48 to 96 hours after the burn, representing a time shortly before the expected death of the burned animal.

The mortality rate in 30 dogs receiving blood from the vena cava of normal animals was 16.6% compared to a rate of 27.7% in 18 dogs receiving blood from the vena cava of animals burned on the lower extremities and distal torso. (Table I) The average drop in blood pressure of dogs receiving blood from normal dogs was 19 mm of mercury compared to a drop of 42 mm in dogs receiving blood from burned animals. There was very little difference in pulse rate in the 2 experiments. Statistical analysis of the data on blood pressure and mortally revealed the figures to be significant although the series is small.

Summary. In our experiments studying the effect of transfusion of blood from a burned animal (dog) to a normal animal we resorted to a method of cross transfusion which obtained blood from the vena cava (draining the burned area), and cross transfused large quantities (several hundred cc) to and from the normal and burned animal. The average drop in blood pressure of dogs receiving blood from normal dogs was 19 mm of mercury, compared to a drop of 42 mm in dogs receiving blood from burned animals in the same quantity and same speed of injection. The mortality rate in 30 dogs receiving blood from the vena cava of normal animals was 16.6% compared to a rate of 27.7% in 18 dogs receiving blood from the vena cava of animals burned on the lower extremities and distal torso.

16064

Certain Mathematical Aspects of the Susceptibility of Erythrocytes to Lysis.*

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Variation in the value of the parametric constant 1/n in the von Krogh alternation formula[1] has been used by several authors[2-4] as a convenient index of the susceptibility of red cells to lysis in the presence of complement and a hemolytic antiserum. It is of interest to consider in some detail the relationship between the value of the constant and the distribution of susceptibility to lysis among individual red cells.

As ordinarily expressed, von Krogh's formula is:

$$x = k\left(\frac{y}{1-y}\right)^{\frac{1}{n}} \qquad (1)$$

where $x =$ lysin concentration
$y =$ fraction of cells hemolyzed
k, 1/n are constants for a given set of reagents.

The plot of y versus x (Fig. 1) is a sigmoid curve which provides an excellent fit for experimental points between the limits $y = 0.1$ and $y = 0.9$. The lack of fit beyond these limits has been attributed to the rapid rate of change of y/1—y as y approaches zero or unity.

Solving Equation 1 for y gives:

$$y = \frac{x^n}{k^n + x^n} \qquad (2)$$

and, differentiating with respect to x,

$$\frac{dy}{dx} = \frac{nk^n x^{n-1}}{(k^n + x^n)^2} \qquad (3)$$

If x is expressed in terms of 50% hemolytic units, then it may be seen from Equation 1 that, at 50% hemolysis ($y = 0.5$), the fraction y/1—y is unity and $k = 1$. This choice of the units of x allows Equation 3 to be simplified to:

$$\frac{dy}{dx} = \frac{nx^{n-1}}{(1 + x^n)^2} \qquad (4)$$

The derivative dy/dx represents the rate of change of the degree of hemolysis with respect to lysin concentration. The value of the derivative at any point x is then proportional to the number of cells that require that concentration of lysin for hemolysis. Thus the curve of dy/dx versus x will provide a graphic representation of the distribution of susceptibility among the red cells of a given population.

The shape of this curve for 3 commonly encountered values of 1/n is shown in Fig. 2. The asymmetry of the curve, which suggests that the lysin is proportionally less effective in the higher concentrations, conforms with Ponder's view,[5] based on experimental data, that the accumulated products of hemolysis have an inhibiting effect on the lysin.

The respective curves for the 3 values of 1/n disclose that there is an inverse relationship between the value of 1/n and the uniformity of a given cell population with respect to susceptibility to lysis. It is suggested that any condition which leads to a change in uniformity will lead to an inverse change in the value of 1/n. This supposition is consistent with the observation of Kent[4] that the optimal degree of cell sensitization with an homologous antiserum occurs at a minimum value of 1/n for the system, and with the disclosure of Morse[2] that 1/n tends to increase either with aging of the cells, or on the introduction of certain impurities.

* Aided by a grant from the Commonwealth Fund.

1 von Krogh, M., *J. Infect. Dis.*, 1916, **19**, 452.

2 Morse, S., Proc. Soc. Exp. Biol. and Med., 1922, **19**, 17.

3 Wadsworth A., Maltaner, E., and Maltaner, F., *J. Immunol.*, 1931, **21**, 313.

4 Kent, J. F., *Science*, 1947, **105**, 316.

5 Ponder, E., *Proc. Roy. Soc.*, B, 1923, **95**, 382.

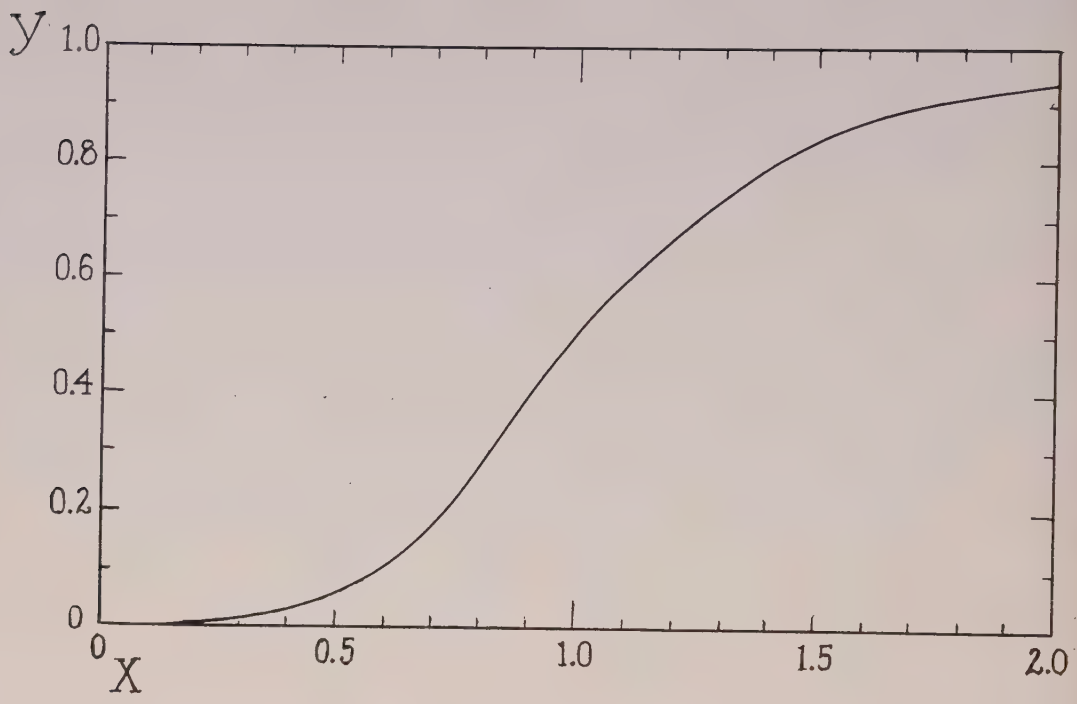
y
1.0
0.8
0.6
0.4
0.2
0
0
X
0.5
1.0
1.5
2.0

FIG. 1.

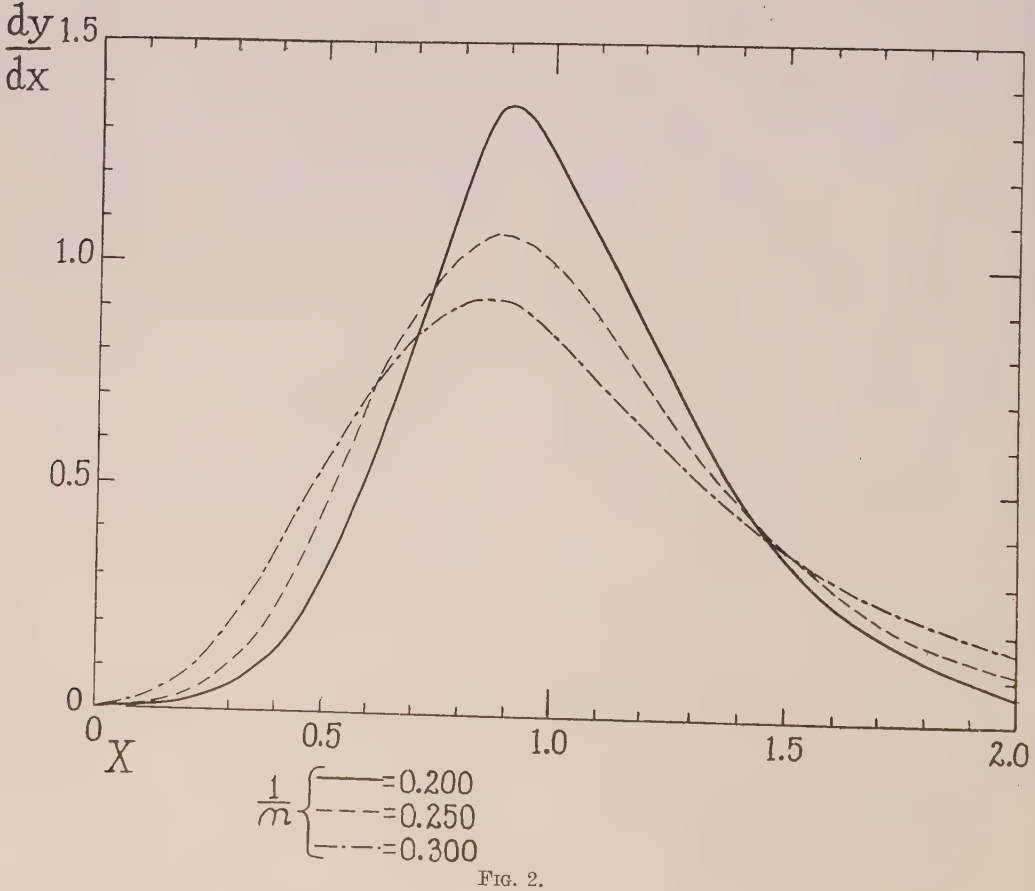
dy/dx
1.5
1.0
0.5
0
0
X
0.5
1.0
1.5
2.0
1/m
=0.200
=0.250
=0.300

FIG. 2.

Summary. An approximate curve representing the distribution of susceptibility to hemolysis among the erythrocytes of a given population is obtained by differentiation of the von Krogh equation. The von Krogh constant 1/n is shown to be an inverse measure of the uniformity of distribution. Variations in the value of 1/n are concluded to be indicative of variations in the uniformity of the cell suspension with respect to its susceptibility to hemolysis.

The assistance of Dr. E. E. Ecker in the initiation and interpretation of this work is gratefully acknowledged by the author.

16065

Bacteria and Cellular Activities. I. Effect of *Streptococcus beta-hemolyticus* on Permeability of Chicken Erythrocytes.*

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In order to obtain a better understanding of some of the changes which may take place in cells under the influence of bacteria and their products, a series of experiments is being undertaken. The initial studies involved changes in the permeability of erythrocytes in the presence of a hemolytic bacterium.

Ponder[1] has reviewed the older literature on this general subject. Many similar experiments have been performed since (Maizels[2]). In all of the work cited interest was centered on the actual hemolysis of the erythrocytes. In the present investigation the permeability of the cells prior to hemolysis was considered.

The following data then are of interest not only with reference to the general problems mentioned, but also emphasize the care which must be exercised to eliminate the possible effect of bacterial contamination in permeability studies of erythrocytes.

Procedure. The test organism, *Streptococcus beta-hemolyticus* was secured from the American Type Culture Collection. It was seeded on proteose No. 3 agar and incubated for 24 hours at 37.5°C. An aliquot of the inoculum removed from the medium by washing with sterile Ringer-Locke solution was added to an equal volume of heparinized chicken erythrocytes obtained by cardiac puncture. This suspension of erythrocytes and bacteria was incubated at 37.5°C. A control suspension was made up in a similar manner except that the organisms were omitted. Aliquots were removed at varying intervals of time, and the permeability of the erythrocytes to glycerol was measured by the photronic cell technique employed by one of the authors (Hunter[3]). Change in volume of the erythrocytes alters the amount of light transmitted to the photocell, hence the current induced and the "scale reading" of the galvanometer. The volumes of the erythrocytes may be routinely varied (1) by placing them in an isotonic solution of a penetrating substance (0.3 M glycerol) which ultimately results in hemolysis or (2) by placing them in a hypertonic solution of a penetrating substance in Ringer-Locke solution (0.3 M glycerol in Ringer-Locke solution), which produces a rapid shrinkage and a subsequent swelling. The light transmission is increased both by swelling and by hemolysis. In the hypertonic solution swelling alone is meas-

* The authors are indebted to the Faculty Research Fund of the University of Oklahoma for grants-in-aid.

1 Ponder, E., *The Mammalian Red Cell and the Properties of Haemolytic Systems,* Berlin, 1934.

2 Maizels, M., *Quart. J. Exp. Physiol.,* 1946, **33**, 183.

3 Hunter, F. R., *J. Cell. and Comp. Physiol.,* 1936, **9**, 15.

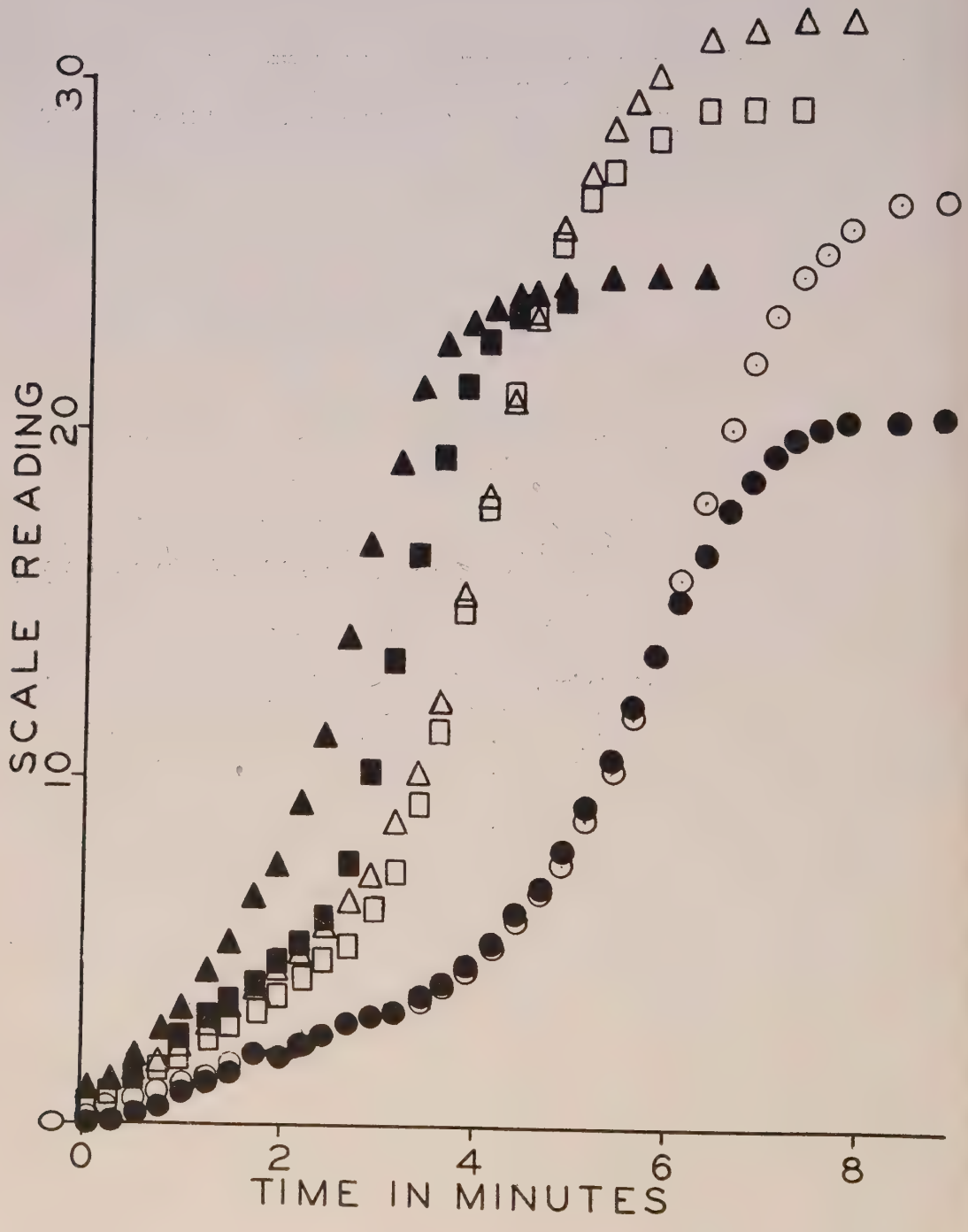

FIG. 1.

Effect of time of exposure to test organism on the hemolysis of chicken erythrocytes in glycerol. ○—control, 0 hr; ●—experimental, 0 hr; □—control, 12 hr; ■—experimental, 12 hr; △—control, 24 hr; ▲—experimental, 24 hr.

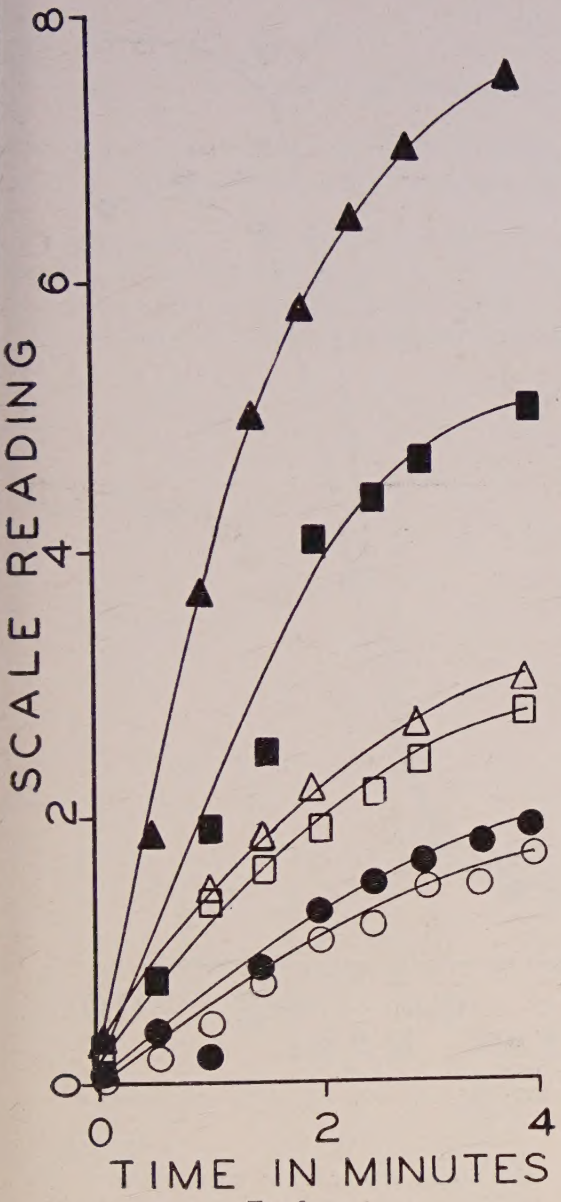

FIG. 2.

Rate of swelling of chicken erythrocytes exposed to test organism for varying times in 0.3 M glycerol in Ringer-Locke solution. ○—control, 0 hr; ●—experimental, 0 hr; □—control, 12 hr; ■—experimental, 12 hr; △—control, 24 hr; ▲—experimental, 24 hr.

ured. In the isotonic solution swelling precedes hemolysis, but since the light changes associated with hemolysis are so much greater than those due to swelling, the hemolysis curves are almost completely a consequence of the loss of hemoglobin from the cells.

Plates were poured to ascertain whether or not contamination had occurred: (1) when the blood was drawn, (2) after the erythrocytes had been centrifuged, (3) at the completion of each experiment, and (4) on the Ringer-Locke solution just before use. Only data from experiments which were free of contamination are considered. After the aliquot had been removed for the permeability studies further sterile precautions were not taken. Contamination introduced during the ten minutes or less required for the measurements would have had a negligible effect.

Results. In general exposure to bacteria for several hours increased the permeability of the cells to glycerol and decreased the time for hemolysis. (Fig. 1) To determine whether or not the change in hemolysis time was actually a consequence of a change in permeability, the rate of swelling was measured simultaneously in a hypertonic solution of 0.3 M glycerol in Ringer-Locke solution. The swelling curves are shown in Fig. 2. The decrease in the hemolysis times varies directly with the increase in the rate of swelling. As a final check fragility measurements were made. Fig. 3 shows that cells exposed to the test organism for 7 hours are not more fragile than control cells.

Discussion. The time for hemolysis and the rate of swelling of the control cells change over a period of many hours. Such a change has been noted in previous work (Hunter[4]), and might well be predicted on the basis of observations such as those of Jacobs and Parpart.[5] Preliminary experiments indicate that this change results at least in part, from volume changes of the cells. It is clear, however, that with erythrocytes the change is more marked in the presence of the test organisms.

Prior to hemolysis a gradual alteration of the cell membrane is induced by the test organism. This is indicated by the change in permeability to the non-electrolyte, glycerol. Hence, in permeability studies in which

4 Hunter, F. R., *J. Cell. and Comp. Physiol.*, 1947, **29**, 301.

5 Jacobs, M. H., and Parpart, A. K., *Biol. Bull.*, 1931, **60**, 95.

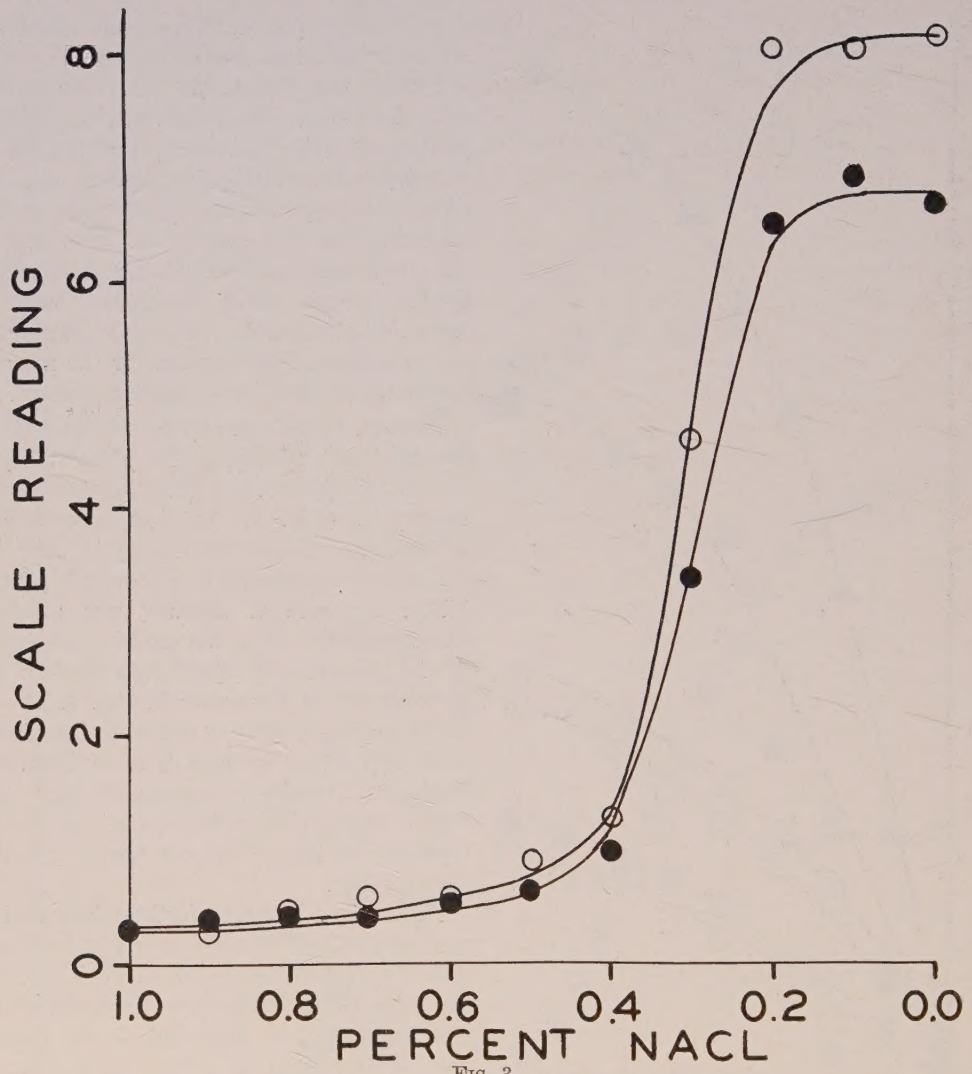

FIG. 3.

Fragility of chicken erythrocytes. ○—control; ●—cells exposed to the test organism for 7 hr.

erythrocytes are maintained for several days under conditions favoring sepsis, the possible effect of bacterial action on the cell membranes must be considered.

Investigations of this nature are being continued to determine the generality of change in the permeable properties of cells under the influence of bacterial toxins.

Conclusions. 1. The permeability to glycerol of chicken erythrocytes exposed to a suspension of *Streptococcus β-hemolyticus* was increased after several hours of exposure to the test organism.

2. Experimental cells were no more fragile than controls.

3. The possible theoretical implications of this work are indicated.

16066

An Improved Device for Recording Activity of Rats.

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Measurement of spontaneous activity of rats can be accomplished satisfactorily by the spring-suspended cage technique of Schulte and associates.[1,2] In these laboratories some improvements have been made which add to the convenience, simplicity and perhaps the accuracy of operation. Essential features of the apparatus are shown in Fig. 1. The spring, cage and work-adder are much the same as previously described. The method of recording is different. Attached to the hub of the work-adder is a lever which can swing freely. This is pulled around by the work-adder wheel until overbalanced. It then falls to trip a mercury switch, completing a circuit momentarily. The switch is connected to a relay which activates a 110 v automatic counter. It is impossible for the falling lever to register more than once for each revolution of the work-adder. The possibility of the contact dipping in the mercury pool 2 or more times per revolution because of slight play in the wheel is thus eliminated. The automatic counter obviates the necessity for tedious or even inaccurate counting of signal marks on a record.

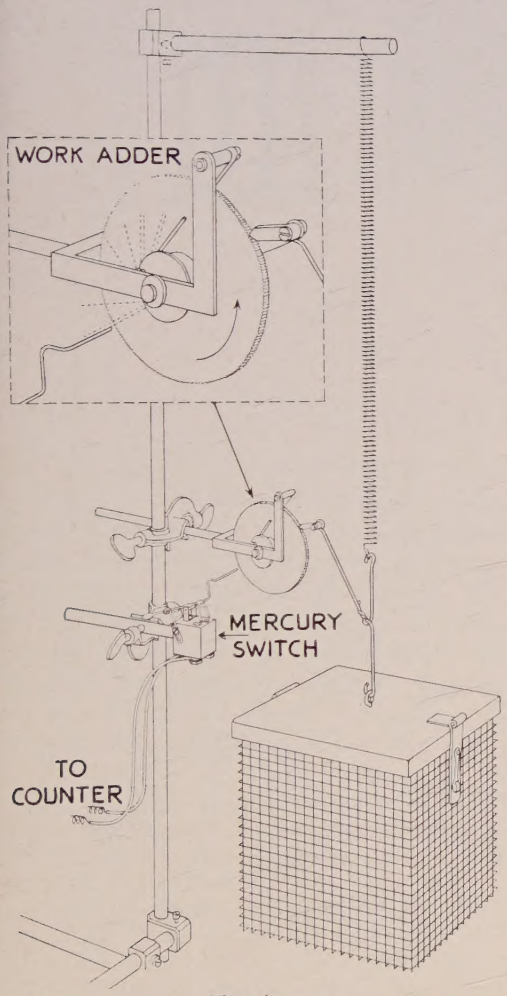

Fig. 1.
Details of apparatus for recording movement of spring-suspended cage.

The long spring which suspends the cage is constructed of steel music wire of 0.029 inch diameter. There are 180 turns in each spring which has an inside diameter of 13/32 inch. Galvanized screen of ¼-inch mesh is used in constructing the cages. The latter weigh about 400 g. For greatest sensitivity, the weight of the cages should be small in relation to animal weight. It has been found convenient to mount 5 complete units of the apparatus on a framework of ½-inch aluminum rods.

In practice, adult rats weighing around 250 g are used. They produce optimal movement for the weight of our cages and the gauge of wire in the springs. There is much variation in the degree of activity of different animals. A selection is made of those which show good activity responses, the others being discarded. The selected rats are used repeatedly with appropriate rest intervals.

For studies of stimulating drugs, constant

1 Schulte, J. W., Tainter, M. L., and Dille, J. M., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 242.

2 Schulte, J. W., Reif, E. C., Bacher, J. A., Jr., Lawrence, W. S., and Tainter, M. L., *J. Pharm. and Exp. Therap.*, 1941, **71**, 62.

light and temperature of the room are required, as suggested by Schulte *et al.* Depressant drugs may be studied in the same apparatus, but the room must be darkened to increase the normal activity. Changes, however, in studies of the latter type are much less striking.

Summary. An apparatus is described for recording the activity of rats in spring-suspended cages. The movements are automatically registered on a magnetic counter.